FUNCTIONAL CHARACTERIZATION OF NLRC5 AND DDX46 IN THE REGULATION OF INNATE IMMUNE RESPONSES

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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May 2014

Major Subject: Medical Sciences

ABSTRACT

The NOD-like receptor protein NLRC5 inhibits TLR-induced NF-κB and viral infection-mediated type I interferon signaling *in vitro*. In the first project, we generated NLRC5 knockout mice to characterize the regulatory function of NLRC5. NLRC5 deficiency enhances Toll-like receptor-induced NF-κB signaling and type I IFN-mediated antiviral immunity in mouse embryonic fibroblasts (MEFs). NLRC5 deficiency also increases proinflammatory cytokine response and antiviral immunity in mouse immune cells. In addition, we confirmed that NLRC5 regulates the transcription of MHC class I gene. Our findings indicate that NLRC5 is a regulator with multiple functions by negatively regulating proinflammatory responses and positively regulating MHC class I gene expression.

The inflammasome is a large protein complex that leads to apoptosis and proinflammatory responses. One of the well-studied inflammasome transduces the activation signal through NLRP3. NLRP3 inflammasome is known to be activated through ASC oligomerization induced by varieties of stimulations; however, the mechanism remains unknown. Some of the DDX family proteins have been reported to sense DNA or RNA to activate type I IFN signaling pathway. By screening DDX family proteins in 293T cells reconstituted with the inflammasome system, we found that DDX46 enhanced NLRP3 inflammasome activity. Knockdown of DDX46 in THP-1 cells reduces inflammasome activity and IL-1β release after crystalline stimulations. DDX46 is cleaved by activated caspase-3 after silica stimulation in mouse macrophages.

Furthermore, cleaved DDX46 interacts with the NLRP-ASC complex following silica stimulation. The positive regulatory role of cleaved DDX46 in silica-mediated NLRP3 inflammasome activation was confirmed in the 293T cell system by its strong ability to interact with ASC. Together, our findings indicate that cleaved DDX46 acts as a bridge to promote the interaction between NLRP3 and ASC after crystalline stimulation.

ACKNOWLEDGEMENTS

I am thankful for people who have been instrumental in helping me all way through my graduate study. I would like to express my gratitude especially to Dr. Rongfu Wang for his unfaltering commitment to my success and pushing me to be my best. Dr. Wang has been a strong, compassionate, and wonderful example of a scientist in the professional world, and I would not have pushed myself through all the difficulties in my graduate training without his support, guidance, and patience. I also appreciate my faithful committee members for their help and their patience in providing structure and insight to my project. I thank Dr. Jun Cui for all of the experimental design and good habits that he handed down to me. I would never have been able to complete these studies without his wisdom and reassuring words. Yinyin Li has been the best classmate. I always remember that she helped a lot in my life when I began the new life here. I would like to extend my thanks to my current and previous lab mates: Qingtian, Ade, Xiaojun, Yanxia, Xiaofan for their help and friendship.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Overview of Immunology

The utmost important task of the immune system is to protect self and respond to nonself. T and B lymphocytes use VDJ recombination to generate precursor cells carrying different T cell receptors (TCR) or B cell receptors (BCR) respectively. Each clone of T cell or B cell is undergoing clonal deletion if the progenitor cell is able to detect self-antigens presented in the thymus or bone marrow. This process is called negative selection (Palmer, 2003). Although other mechanisms such as anergy (Hammerling et al., 1991) and receptor editing(McGargill et al., 2000; Wang et al., 1998) exist in the periphery to induce tolerance to self, the central tolerance is considered to play a major role to select or train T cell and B cell clones to react only to nonself- antigens. The discovery of the theory of negative selection won the Nobel Prize for Burnet and Medawar in 1960 and ever since the self-nonself discrimination model has played a dominant role for several decades.

But the major problem of self-nonself model is that not only *in vitro,* but also *in vivo* studies showed that a robust immune responds for T cells need co-stimulation signals. The recognition of nonself-antigen alone is not sufficient to induce adaptive immune response. For example, injection of ovalbumin antigen subcutaneously, intravenously, or intraperitoneally will not elicit an immune response against it unless a

second agent is applied which is called adjuvant. One of the most commonly used adjuvant is complete Fraud's adjuvant (CFA) which contains inactivated and dried mycobacteria in mineral oil. In 1989, Charles Janeway suggested an ingenious model suggesting the capability of antigen presenting cells (APCs), such as macrophages, dendritic cells to recognize a bunch of evolutionarily conserved pathogen molecules which are referred as pathogen-associated molecular patterns (PAMPs) from microbial pathogens such as bacteria and viruses. Upon recognition of these PAMPs by a set of receptors encoded in germline by APCs, they are able to up-regulate co-stimulatory molecules and co-stimulatory cytokines to facilitate T cell activation. The discovery of Toll-like receptors, which represents one crucial member of pattern recognition receptors (PRRs) is rewarded for the Nobel Prize in 2011 for Physiology or Medicine. According to the infectious-nonself model the T cells will only respond to non-self only if there is innate immune system providing appropriate stimulatory signals upon recognition of PAMPs. Thus, the choice of responding or not responding to antigens is mainly depend on the innate immune receptors when they encounter appropriate stimulation such as LPS.

Innate Immunity

Innate immune system acts as a sentinel of the mammalian body; it senses or recognizes microbial pathogens the instant they invade the body, and defends the pathogens through phagocytosis or pro-inflammatory responses. A wide variety of cells

contribute to the cellular composition of the innate immune system. It involves almost all the white blood cells except for T and B cells. It also involves epithelial cells such as skin, gut and lung cells that line our internal area of the body. The innate immune system can directly prevent bacterial invasion through cellular engulfment of pathogens and digesting bacteria within cells. Neutrophils and macrophages are two of the well-known immune cell types that have the ability to clear infections through this mechanism. In tissue epithelial cells, they can secret antimicrobial peptides to combat microbes. Unlike adaptive immune system, innate immune response is non-specific to the pathogen, and does not maintain the memory for defending the infection. In addition, compared to the later emergence of adaptive immunity, the innate immune system exists in not only vertebrates, but also in invertebrates and plants which are evolutionary more ancient species. Considering the prosperous of species of plants and invertebrates, the innate immune system's success is out of doubt. The appearance of adaptive immunity indeed added a lot of specificity of immune responses due to the TCR and BCR recombination and selection in the central immune organs. Yet, adaptive immunity also brought up problems such as autoimmune diseases and allograft rejection. Accordingly, the adaptive immunity is still tightly regulated by co-stimulatory molecules. Inflammatory cytokines are also acting as a co-stimulatory factor to activate the adaptive immunity. The upregulation of co-stimulatory molecules and proinflammatory cytokines requires the NF-κB signaling which is a key player in innate immunity. Finding regulators of the NF-κB signaling pathway is very important to understand and intervene such pivotal signaling.

Pattern Recognition Receptors (PRRs)

PRRs are receptors which can detect or sense conserved molecule from microbial called pathogen associated molecular patterns (PAMPs) and they are either in the cytoplasm or on the cell surface. Once the host is challenged by a potential infection or factors regarding as “danger” signals which are present in response to pathogenic infection, cell stress, or sterile inflammation, the PRRs sensing the infectious molecules or danger signals, could induce a cascade of signaling pathways that involves assembly of adaptor molecules, activation of kinases and transcriptional factors, and ultimately lead to the upregulated gene expression of important molecules, such as cytokines, adhesion molecules and co-stimulatory molecules. These effector molecules result in the initiation of immune response and mount an effective adaptive immune response. Several classes of PRRs are characterized such as TLRs (Toll-like receptors) which is the earliest one identified RLRs (RIG-I-like receptors) that mainly detect viral infection and NLRs (NOD-like receptors) which play multiple functions in immune system.

Toll-like Receptors (TLRs) and NF-κB Activation

Ten members of TLRs have been characterized in human. They are distinguished from each other by their ligand specificities. TLRs can bind to a variety of evolutionarily conserved pathogenic molecules such as Lipopolysaccharide, peptidoglycan, flagellin, CpG or unmethylated cytosine-guanine dinucleotide or

sequences from the genome of bacteria or virus, zymosan from fungi, dsRNA and ssRNA. Some TLRs needs additional PAMP-binding molecule to recognize the ligand, for example, LPS recognition by TLR4 is mediated by an accessory molecule named MD2. TLRs can act as both PAMP and DAMP sensors. For instance, besides LPS, TLR4 can also recognize structurally and biochemically diverse ligands such as the fusion protein from virus such as respiratory syncytial virus and some heat shock molecules. Upon activation by ligand binding, TLRs trigger a common signaling pathway that activates NF-κB signaling pathway. Take TLR4 as an example, once the ligand LPS is bound to the receptor, the adaptor protein Myd88 is recruited (Medzhitov et al., 1998; Muzio et al., 1998). The C-terminal of Myd88 is responsible for interaction with the TLR complex and the N-terminal is responsible for recruiting downstream adaptor protein IRAK4 (Wesche et al., 1997). IRAK4 is a serine threonine kinase which can autophosphorylate itself and recruit TRAF6. TRAF6 has a RING finger domain which acts as an ubiquitin E3 ligase and promotes the assembly the K63-linked ubiquitin chains. These ubiquitination chains provide interaction sites where TAK1/TAB1/TAB2 complexes are formed (Xia et al., 2009). The complex phosphorylates IKKβ and activates IKK complex phosphorylates, resulting in the degradation of IκB which is the inhibitor of nucleic transportation of NF-κB transcription factor. Thereby, P65 and RelA is released and transferred to the nucleus and activate the downstream target gene expression (Muzio et al., 1998). NF-κB activation plays a central role in bridging the innate immune responses with effective adaptive immune responses because it mediates and co-operates the expression of multiple genes that are essential to mount effective

immune response. The NF-κB regulated genes include genes transcript pro-inflammatory cytokines such as pro-IL-1β, IL-6 and TNF-α; genes encoding chemokines such as IL-8 and RANTES; genes encoding cell adhesion molecules; and those transcript co-stimulatory molecules such as B6 family members CD80 and CD86. These effector molecules together promote the phagocytosis of microbial pathogens, enhance killing of target bacteria utilizing complement or NK cells, and promote antigen presentation and recognition. Moreover, the NF-κB signaling pathways are often associated with cellular growth and apoptosis inhibition, thus is promotes the expansion and persistence of cellular immunity. The activation of NF-κB as described is obviously a complex multistep signaling cascade which can be targeted at different levels.

CHAPTER II

FUNCTIONAL CHARACTERIZATION OF NLRC5 IN THE REGULATION OF INNATE IMMUNE RESPONSES*

Introduction

Regulation of NF-κB signaling

Because uncontrolled activation of NF-κB signaling will cause secretion of large amounts of inflammatory cytokines which will cause uncontrolled activation of T cells leading to autoimmunity or overwhelming cytokine storm can cause septic shock and death of the organism. In this regard, regulating, especially negative regulation of NF-κB signaling pathway is truly critical in balancing effective immune response versus uncontrolled inflammation and tissue damage. Generally speaking, the degradation of adaptor molecules in the signaling pathway is a basic mechanism of terminating an activation signal. In mammalian cells, the degradation of a particular protein is usually mediated by proteasome which recognizes K48 poly-ubiquitination modification. A typical example of degradation of TLRs is Triad3A which is a protein containing RING finger domain. Traid3A was initially discovered by yeast-two-hybrid of TLR9 TIR

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domain and was later proved to be interacting with multiple TLRs such as TLR3, TLR4. Overexpression of Triad3A caused the degradation of interacting TLRs (Chuang and Ulevitch, 2004).

Of course, K48-polyubiquitination is not the only way to negatively regulate NF-κB signaling pathway, there are other mechanisms involving ubiquitination. It is notable that K63 polyubiquitination is very critical in NF-κB activation, thus, de-ubiquitinate K63 polyubiquitin chain can be efficient ways to shut off the signaling. The well-known example of this class is the discovery of deubiquitinase A20. A20 is a Zinc-finger protein, which is induced by TNF. A20 can also act as a negative feedback loop though its regulatory function, to regulate TNF induced NF-κB activation. The target of A20 is RIP1, which is a critical adaptor protein in TNF induced signaling pathway. Deubiquitinate K63 poly-ubiquitin chain of RIP1 cause its loss of function to conduct activation signals downstream (Sun, 2008). Indeed, A20 deficient mice have profound systematic inflammation. But crossing A20 KO mice with TNFR KO mice cannot rescue all the phenotype indicating that A20 has targets other than RIP1. Later, it was found that N-terminal OUT-like domain of A20 can remove K63 poly-ubiquitin chain on TRAF6 and shutdown TLR induced NF-κB activation as well (Boone et al., 2004).

Another deubiquitinating enzyme called deubiquitinating enzyme A (DUBA) is also an important negative regulator of deubiquitinase. DUBA and A20 belong to the same family consisted of 14 members. The signature motif of the family is OUT domain. DUBA targets TRAF3 and prevent the autoubiquitination of TRAF3 (Kayagaki et al., 2007).

Another family of deubiquitinase is USP (ubiquitin-specific protease) family. One important member of this family is CYLD. CYLD has multiple target proteins such as TRAFs, NEMO, Bcl3, TAK1 and RIP1. Knockdown of CYLD could increase both NF-κB and p38 MAPK activity (Yoshida et al., 2005). Another common mechanism of negative regulation is competitive binding of adaptor proteins. As previously stated, the TLR signaling pathway is conducted by proteins using similar domain for interaction, for example, TIR domain. Adaptor proteins: Myd88, Mal, TRIF, TRAM all have the TIR domain to mediate interaction and assembly of complexes. SARM is a protein which also has TIR domain but does not mediate positive signaling. Thus by competing with signaling molecules, SARM is using its TIR domain to sequester NF-κB activation. Knockdown of SARM could significantly enhance LPS induced cytokine production (Carty et al., 2006). At the receptor level, RP105 (Radioprotective 105) is highly homologous to TLR4. Like TLRs, RP105 is rich of extracellular LRR repeats but no TIR structures. Thus, RP105 could compete with TLR4 to bind to MD2 and form TLR4/MD2 complex, thereby negatively regulating TLR4 signaling (Divanovic et al., 2005).

Another important mechanism to generate competitive binding proteins is by alternative splicing. Until now, many adaptive proteins in the TLR signaling pathways have been discovered to have alternative splice forms to compete with full length adaptive proteins. One important example is the discovery of Myd88s. Myd88 is a crucial adaptive protein in all of the TLR mediated signaling except for TLR3. It mainly has three structural domains: 1) N-terminal DD domain, 2) middle domain and 3) TIR

domain. Myd88s, one isoform of Myd88, does not contain the middle domain and inhibit the activation of TLR signaling pathway. Evidences showed that exogenous expressed Myd88s inhibits TLR-induced NF-κB signaling, because Myd88 and Myd88s could form a heterodimer and recruit IRAK1 but fails to phosphorylate IRAK1, thus shutting down the signaling (Burns et al., 2003; Janssens et al., 2002).

RIG-I like receptors and type I IFN pathway

TLRs are mainly expressed on the cellular membrane system including cell surface membrane or membrane system of endosome or lysosome, they are not capable to recognize intracellular cytosolic pathogens that are not involving endocytosis. In addition, in TLR3/TLR9 or even TRIF/Myd88 double knockout mice, the mice are still able to induce type I IFNs upon viral infections. This strongly indicates that other TLR independent recognition of intracellular molecular patterns is needed to the signaling activation. Generally speaking, virus infection can trigger IRF3 or IRF7 activation to produce type I IFNs. Type I IFNs includes IFN-β and several subtypes of IFN-α, which are very crucial for host cells to combat the virus infection and restrain the virus reproduction. Until now, at least two types of PRRs are regarded to recognize viral PAMPs. One is toll-like receptor as described above; the other RIG-I like receptors (RLRs). This family of receptors so far has been identified to include RIG-I, dicer 2 and MDA5 (melanoma differentiation-associated gene 5). They are responsible to recognize virus-differentiated PAMPs. Kato and his colleagues demonstrated that RIG-I KO MEFs are defective to respond to some kinds of viruses such as type A influenza virus, JEV

(Kato et al., 2006), whereas MDA5 KO MEFs responds normally to these viruses. In contrast, MDA5 KO MEFs cannot respond to small RNA viruses such as EMCV, whereas the antiviral response in RIG-I KO MEFs is normal when infected by these viruses. Structural analysis of RIG-I revealed that the N terminus CARD domain is important for activation. The C terminus region masks the N terminus CARD domain when the protein is inactivated. Upon activation, the CARD domain of RIG-I is exposed and easily to form dimers and recruit MAVS (mitochondrial antiviral signaling), to activate type I IFN secretion pathway sequentially through signaling molecules TBK1, IKKi, IRF3 and IRF7. Recently, stimulator of IFN genes (STING), mainly function as an adaptor protein was described by a different group. Although STING is proved to be present on the mitochondrial membrane, it can also interact with RIG-I and MAVS, providing critical links to the cross talk of signals from both the mitochondria and ER. In one study, the HSV-infected mouse macrophages generated a lot of mitochondrial ROS and in response to the alternation of ROS concentration; calcium release from the ER was further activated. This response then lead to the activation of P38, JNK or NF-κB activation through TAK1 mediated signaling (Mogensen et al., 2003). In the RLR signaling pathway, MAVS is proved to be the divergent point of IRF transcriptional factors and NF-κB activation. TNF receptor-associated death domain (TRADD) is recruited to mitochondrial protein MAVS and form a complex with MAVS. TRADD then recruits the TRAF3 and TANK to activate the TBK1/IKKi complex. And then the activated complex phosphorylates IRF3/IRF7, leading to the transcripti on of IFN-β(Guo and Cheng, 2007; Kato et al., 2005; Michallet et al., 2008; Saha et al., 2006;

Yoneyama et al., 2004). In the meantime, by utilizing the Fas associated death domain, the MAVS/TRADD complex also activates the signal to NF-κB based on the interaction between this complex and RIP1 and caspase8. These complexes activate the IKK complex and lead to NF-κB activation.

Virus evasion strategies and downregulation of type I IFN pathway

Viruses, during the evolution and selection by environment, have developed advanced mechanism to interfere the antiviral responses, particularly the production of type I IFN. Generally, viruses are able to block functional molecules which are participating in the activation of NF-κB or IRF pathways to inhibit the production of IFNs. One of the immediate early proteins generated by viruses such as HSV-1, for instance, infected-cell protein o (ICPo) negatively regulates the activation of IRF3 and IRF7 (Lin et al., 2004). Similarly, NS1/2 of RSV inhibits IRF3 phosphorylation mediated by TBK1 (Spann et al., 2005). Likewise, measles virus inhibits phosphorylation of IRF7 (Schlender et al., 2005). The most extensively studied molecule is NS3/4 serine protease bio-synthetically produced and released by hepatitis C virus. It can cleave adaptor molecule TRIF to inhibit TLR3 responses and also cleave MAVS to prevent RLR-mediated signaling pathways and the signaling activated production of antiviral proteins called type I IFNs (Li et al., 2005; Meylan et al., 2005). Some paramyxoviruses can inhibit the MDA5 recognition by the viral V protein (Andrejeva et al., 2004). VP35 of Ebola virus and NS1 of influenza A virus inhibits the interaction between RIG-I and MAVS after the viral RNA is recognized by RIG-I. (Cardenas et al.,

2006; Mibayashi et al., 2007). Recently, another mechanism of avoiding RIG-I recognition has been described. Some viruses such as Crimean-Congo HFV, Hantaan virus and Borna disease virus can remove of 5'-triphospate RNA from their genome RNA post-transcriptionally to prevent RIG-I binding and activation (Habjan et al., 2008). Finally, IFNs once have been transcribed, viruses have also developed post-transcriptional strategies to prevent the cytokine from being processed. For example, VSV M protein can inhibit the export of the transcript of IFN from the nucleus to the sites for translation in the cytoplasm (Her et al., 1997). In addition, HSV-1 can destabilize proinflammatory cytokine mRNAs by ICP4 and ICP27, which are viral immediate early proteins (Mogensen et al., 2004).

Although the innate immune response to virus stimuli is essential, over reaction to such stimuli can do harm to the host as well. Thus, strict regulations of type I IFN pathways are needed. There are a lot of negative regulators have been found to participate in down-regulating the pathway such as A20, Pin1, SIKE, RNF5, DUBA and DAK (Diao et al., 2007; Huang et al., 2005; Kayagaki et al., 2007; Lin et al., 2006; Saitoh et al., 2006; Zhong et al., 2009). Different molecules utilize different target and strategy to act as negative regulators of the type I IFN pathway. Here we found NLRC5, which belongs to a Nod-like receptor superfamily, can regulate type I IFN pathway at the receptor level and by generating KO mice, we are going to validate its role *in vivo* by infecting the mice with VSV or influenza viruses to analyze the physiological function of NLRC5 in the regulation of type I IFN pathways.

Nod-like receptors (NLRs)

The general paradigm of extracellular bacteria PAMP recognition about a decade ago is mainly through the TLRs as introduced above. But in the past 10 years, a group of intracellular receptors: NOD-like receptors are found to be one of the PRR families that can recognize PAMPs. NLRs share a typical tripartite structure including a conserved domain capable to bind to nucleotide, NACHT. They also contain a C-terminal formed by a number of leucine-rich repeats (LRRs), which is estimated to be able to sense PAMPs, and is named LRR domain. In addition, some NLRs have a pyrin domain (PYD) in the N-terminus, while some may contain a caspase-activation and recruitment domain (CARD). Until today, there are about 23 characterized NLR family members playing a role in cellular processes such as pathogen recognition, apoptosis, gut development and inflammasome activation(Kufer and Sansonetti, 2011). Three NLR are identified: 1) the NLRC family which has CARD domains in its N-terminal, 2) the NAIP family which has BIR domain in the N terminus of the protein, and 3) NLRP family which has PYD domain as a specific N terminal domain. NLRP family contains 14 family members. Two of the NLRC proteins, NOD1 and NOD2 were reported to be capable to recognize of bacteria (Strober et al., 2006). Among the NLRP family members, NLRP3 is the most extensively studied member which plays an important role in activation of inflammasome (Tschopp and Schroder, 2010). Functions of other NLRP members were also studied recently. For example, NLRP1 can form inflammasome after sensing of Bacillus anthracis toxin(Levinsohn et al., 2012). NLRP4 is another NLRC protein that has been reported to be a negative regulator of virus-induced type I

interferon signaling via degradation of TBK1(Cui et al., 2012). NLRP6 negatively regulates TLR signaling pathway and promote the secretion of mature IL-18 through inflammasome activation to promote tissue repair in the intestine (Anand et al., 2012; Elinav et al., 2011). NLRP7 is also important for inflammasome activation when the lipopeptides from pathogens are sensed by the receptors (Khare et al., 2012). NLRP10 is reported to induce dendritic cell activation and initiate adaptive immunity through cytokine induced regulations (Eisenbarth et al., 2012); Pathogenic molecules from the Yersinia pestis can be recognized by innate immune system and activate NLRP12 inflammasome (Vladimer et al., 2012) as well as suppress colon inflammation and tumorigenesis by regulating NF-κB signaling pathway (Allen et al., 2012; Zaki et al., 2011). Thus the functions of NOD-like receptors vary from modulating NF-κB signaling, recognizing pathogens, and regulating adaptive immunity, to activating tumorigenesis and inflammasome.

NLRC5

Human NLRC5 consists of 1866 amino acids and mouse NLRC5 consists of 1915 amino acids. They are highly conserved, 64% of the amino acids are identical between human and mouse NLRC5. It is the largest protein among the five NLRCs in the family. The C-terminal LRR domain is extremely long, containing 27 LRRs. The homologous alignment shows that NLRC5 is mostly close to CIITA which can be transported into nucleus directed by the nuclear localization signals (NLSs) (Benko et al., 2010; Meissner et al., 2010) and regulating MHCII gene transactivation (Camacho-

Carvajal et al., 2004). In addition, the N terminus of NLRC5 protein is also found to have NLS, suggesting a role of NLRC5 in the nucleus. In fact, overexpression of NLRC5 is truly found to be mainly localized in the nucleus whereas endogenous NLRC5 is found to be in both nucleus and cytoplasm. Inhibition of NLRC5 to be transported into cytoplasm can be achieved by LepB, which is an inhibitor of protein nucleus export (Neerincx et al., 2010; Staehli et al., 2012).

Expression of NLRC5 is widely detected in cells and tissues, and the expression level of the protein in the spleen, lymph node, and other organs important for immune response is found to be especially which suggests its function in immunity (Cui et al., 2010). Moreover, NLRC5 expression is inducible in response to stimuli such as type I IFN, type II IFN and LPS (Cui et al., 2010; Staehli et al., 2012; Yao et al., 2012). Interestingly, the induction of NLRC5 in response to LPS does not signal through adaptor protein Myd88 but through TRIF suggesting that LPS-induced expression of NLRC5 may due to the type I interferon release when TLR is activated (Staehli et al., 2012). People tried to analyze the interactive ability of promoter region of NLRC5 with transcription factors by CHIP-Sequencing and found that both STAT1 and NF-κB have putative binding site with NLRC5 promoter (Kuenzel et al., 2010) , thus to determine exactly how NLRC5 is regulated is needed by utilizing genetic models such as STAT1 KO mice.

Function of NLRC5 in innate immunity

Previous studies have reported that NLRC5 plays a negative regulatory role in the signaling pathway of NF-κB activation and anti-viral response (Benko et al., 2010; Cui et al., 2010; Neerincx et al., 2010). In the NF-κB signaling pathway, NLRC5 interacts with IKKs and mediates K48 ubiquitination and degradation to shut down the NF-κB signaling pathway, whereas in type I interferon producing pathway, NLRC5 is suggested to prevent activation of type I IFN signaling by interacts with RIG-I like receptors (Cui et al., 2010). Nevertheless, whether NLRC5 is playing such roles *in vivo* is not clear. Here, by constructing a knockout mouse by site-specific targeting exon 8, we are able to characterize the role of NLRC5 *in vivo* in primary mice immune cells such as MEFs, peritoneal macrophages, BMDCs, BMMs and at the whole mouse level.

Materials and Methods

Generation of NLRC5-deficient mice

In the cDNA of mouse *NLRC5*, the eighth exon encodes partial region of the gene expressing functional domain of the protein, called LRR1domain. We constructed the targeting vector and removed exon 8 through homologous recombination at the region where the target gene and the constructed vector share identical sequence. And the insert of constructed sequence with a Neo cassette resulted in incomplete transcription of NLRC5. The linear fragments of targeting vector were transfected into embryonic stem (ES) cells by electroporation. Then we did PCR and Southern blot to

verify the homologous recombinant stem cells. The ES cells were selected with neomycine to get the cells undergo recombination. Simultaneously, the cells with non-specific recombination were killed by the treatment of gancyclvir. The ES cells, after selection, were put back into blastocysts. And then they were put into several embryos of white-fur mice. If a mouse is with the genes after recombination in the skin cells, it would appear as chimeric mouse. Using the chimeric mice for breeding, we were expecting to get some gray-fur mice, which is heterozygous for NLRC5 knockout allele. Grey mice were used to set up breeding pairs to give birth to homozygous *NLRC5* knockout mice.

Cell culture and stimulation

Mice were sacrificed around day 15 of pregnancy to generate Mouse embryonic fibroblasts (MEFs). The embryo tissues were cut into small pieces, and then we treated the cells with trypsin to separate the link between MEF cells by degrading the collagen between cells. The single cell suspension were then washed to remove the reagents and cultured in MEF medium.

Peritoneal macrophage extraction: each mouse was injected with 3 mL thioglycollate medium through intra-peritoneal injection 4 days before cell harvesting day. On the cells extraction day, mice were sacrificed by CO2 and cervical dislocation, then the fur covering the abdomen were dispelled from the peritoneal. Inject 10 mL of 3%FBS/PBS into the peritoneal cavity, massage the abdomen for 20 times by gently shaking, and collect the lavage with cells by syringe. Cells were allowed for attaching in

the petri dish with culture medium for 3 h, and the floating cells after that were removed by washing with PBS for three times. The attached cells were collected and seeded for experiment.

Generation of bone marrow-derived macrophages (BMM) and dendritic cells (BMDC): tibia and femur were removed from the sacrificed mice, and the flesh was removed using disinfected scissors. Cut both ends of the bones and flush out the bone marrow using syringe with RPMI medium. Cells were treated with Red blood cell lysis buffer and washed with medium for twice, and then the resuspended cells were counted for seeding. Cells were seeded with RPMI1640 containing 10% FBS, 55 μM β-mercaptoethanol, 1% penicillin-streptomycin, and appropriate amount of L929 conditioned media for 4 days for the bone marrow progenitor cells to differentiate into BMMs. Or the progenitor cells were cultured with RPMI 1640 supplemented with 10 ng/ml mouse GM-CSF, and allow the cells to differentiate in to BMDCs for at least 7 days. In the cell stimulation experiments, mouse macrophages and dendritic cells were treated with TLR4 specific ligand LPS (100 ng/ml), TLR2 specific ligand Pam3CSK4 (1 μg/ml), TLR9 specific ligand CpG (2 μg/ml), TLR7 specific ligand CL-097 (1 μg/ml), TLR3 specific ligand polyinosinic-polycytidylic acid (poly(I:C)) (20 μg/ml) for indicated time points. LPS is produced by Sigma-Aldrich. Other TLR agonists such as CpG, poly (I:C) are the products of Invivogen.

Flow cytometry

For the analysis, the cells are stained with Fluorescence conjugated antibodies All these antibodies were the products of BD Pharmingen. Before staining, cells were purified by positive or negative selection so that the cells are not damaged during analysis on the flow cytometry and also permit a lower background. Purified cells were washed with staining buffer. We used 1%FBS/PBS with 0.1% Azide to be isotonic and buffer to neutrality, meanwhile cushion the centrifugation pressure, block non-specific binding, prevent and block Fc receptor binding. Adjust density of cell suspension to about 5 million cells/ml. The number of viable cells is expected to be no less than 90% of the total cells, measured by trypan blue exclusion. Cells were centrifuged at 400 rpm for 5 minutes. The medium were carefully aspirated, and the cells were stained with 100ul of first antibody diluted in the staining buffer. Incubate the staining samples for 30 min at 4^{o}C in dark. Wash the cells three times after staining. After wash, the stained cells were flicked and resuspended with100ul of secondary antibody if necessary. After that, we resuspended the stained cells with 100ul of staining buffer and transferred the mixture to FACS tubes containing 400ul staining buffer for analysis. Cells need to be placed on ice when handling.

Cytokine release analysis

In our study, to treat the cells, MEF cells or macrophages were incubated in the medium with indicated stimuli. The cell culture supernatants at different time point were collected and analyzed for cytokine release with commercial ELISA kits which detect

indicated cytokines we studied in this project. These kits were purchased from eBiosceince and IFN-β detecting set is from PBL Biomedical Laboratories. Analysis was carried out according to manufacturer's instructions. To challenge the mice *in vivo* such as LPS injection, we used a dose of 25 mg/kg for intraperitoneal injection, and then we collected sera of the mouse at indicated time points after injection. The serum was analyzed for the release of IL-6 and TNF-α. To do the *in vivo* challenge of virus, mice were intravenously injected with VSV-eGFP. And the type I interferon release in the sera were analyzed using ELISA.

ELISA

Sample preparation: Cell culture supernatants were collected into 1.5 mL tubes at different time points as indicated. To remove the cell debris or any other residue of the reagent of treatment in order to avoid any factors that affect the ELISA efficiency, centrifuge the tubes at 12,000 g for 5 min, the supernatant after centrifugation were collected in a new tube. For stocking, freeze the samples in -80^{o}C so that the bioactive molecules are not degraded. ELISA plate preparation: Dilute capture antibody in PBS as instructed, use 100 ul of the diluent for each well, and coat the plate for first antibody binding in 4 degree overnight. On the second day, wash the plate with 0.5% Tween/PBS for three times, and incubate each well with 200 ul of 1%BSA/PBS for 2 hours to block the non-specific binding site on the plate. Prepare sample diluents and standard gradient samples with sample diluents buffer, and add 100 ul sample diluents to each well. The loaded plate are then incubated in dark for 2 hours, followed by a 3-time wash. Incubate

each well with detection antibody diluents for 2 hours, followed by 0.5 hour incubation of HRP. Wash the plate for 5-6 times with the wash buffer, and add 100 ul of biotin substrate to initiate the reaction. Then we use 50 ul stop buffer to terminate the substrate reaction. The absorbance was measured using plate reader. The raw data were analyzed using Excel to generate the standard curve and to calculate the concentration of cytokines in the supernatant before dilution.

Total RNA extraction

Experimentally treated cells were treated with Trizol (invitrogen), mix the lysate thoroughly to homogenize the lysate. 100 ul of chloroform were added to each 500 ul of Trizol lysate. Vortex the mixture to completely mix the substances. Sit the tube at room temperature for 5 min, and then centrifuge the samples at 12,000 xg for 15 min. Carefully collect the supernatant after centrifuging, and add equal volume of isopropanol, mix thoroughly and sit in room temperature for 7 min and then centrifuge the samples at 12,000 xg for 10 min. The RNA pellet was washed for 2 times with 75% ethanol to remove the organic diluents. Then, dry the pellet on dry tissue by inverting the tube for 15 min. Dissolve the RNA pellet using molecular grade water then the pellet appears to be transparent. Pipet the diluents up and down to dissolve the RNA completely, incubate the tube in 65 centigrade to accelerate dissolution if necessary. We measured RNA concentration after complete dissolution.

Western blot

Before collecting the treated cells, we washed the wells with PBS and then add low salt lysis buffer or RIPA buffer to lyse the cells and release the protein. For attaching cells, we used cell scraper or trypsin to release the cells from the bottom, and then collect the cells with Eppendorf tubes and add lysis buffer. The samples were inverted in the 4 degree shaker for 20 minutes for the protein released from the cytoplasm and then spun down. Then we stock the supernatants for analysis. The resulting lysate was first measured for protein concentration by BCA protein quantification protocol and then frozen for later use. Upon western blot analysis, the protein lysates were diluted to the equal total amount of protein with the lysis buffer and then mixed with SDS-loading buffer. We then boiled the mixture of lysate and loading buffer for 5 minutes at 100 ℃, and then loaded the denatured protein samples onto SDS-PAGE gel to run western using 80 V for to stack the protein in the upper layer gel, and later at 120 V to separate the protein in the lower layer gel. After this step, the gel was transferred on to a nitrocellulose membrane using wet transfer system at 100 V for 90 min. Then the membrane was blocked and blotted with indicated antibody using gel imaging system.

Serum collection

Blood collection from mouse tail: Warm the mouse and put it into a restraining tube. Rub the tail from bottom to tip, so that the blood flow does not increase. Using a straight edge razor quickly remove about 1 cm of the tail. We used a capillary tube to

suck the blood and transferred the blood to tubes. Apply pressure to stop the bleeding after collecting the blood. In our case, several samples are needed for different time point after challenge within several hours; we removed the clot to open the existed wound to collect blood samples. Allow the blood to clot for 30 min at ambient temperature (19-24℃) , and then Centrifuge the sample for 10 min at 2200 G. Separate the serum and plasma by removing the transparent supernatant after centrifuging. Pipette aliquots of samples for several separate uses and store samples at -80 ℃, make sure to avoid freeze-thaw cycles to keep the concentration of bioactive cytokine and chemokines at the original level.

Virus titration

Seed $2x10^4$ cells in each well of 96-well plate. One the second day, dilute the virus concentrate with medium by 10 time for 10 times. Remove the medium in the well, and add 120 ul of diluted virus into each well. Use duplicate for each dilution. Exam the infected cells 24 hours after infection under fluorescence microscopy to detect the infection of eGFP tagged virus infection. Then, the virus titer was determined.

Results

NLRC5 induction is dependent on Stat1signaling pathway

NLRC5 expression is reported to be induced by cytokines such as IFN-β and IFN-γ, as well as TLR ligands. However, the mechanism how NLRC5 is induced has not been studies yet (Benko et al., 2010; Cui et al., 2010; Kuenzel et al., 2010; Neerincx et al., 2010). Previous study of our group showed that LPS can induce NLRC5 expression in mice macrophages, but we are not clear whether LPS induce NLRC5 directly through TLR-induced NF-κB signaling pathway or through other mechanisms. To test whether NLRC5 is induced by NF-κB, we examined NLRC5 mRNA transcription in response to LPS or IFN-β stimulation in mouse peritoneal macrophages of $MyD88^{-/-}$ and $IRAK4^{-/-}$. LPS induced TLR4 signaling pathway is dependent on Myd88 and Irak4 to activate NF-κB signaling. Thus, $MyD88^{-/-}$ an $Irak4^{-/-}$ cells will not be able to activate LPS-induced NF-κB signaling. When we treated the $MyD88^{-/-}$ or $IRAK4^{-/-}$ macrophages with LPS, we found that the induced expression of NLRC5 mRNA was completely shut down in these cells (Figure 1A), indicating that LPS induced NLRC5 relies on Myd88 and Irak4 dependent NF-κB pathway. However, NLRC5 induction in response to IFN-β treatment in absent of Myd88 or Irak4 was comparable to the induction pattern in wild-type (WT) cells, suggesting that IFN-β induce NLRC5 expression is independent of Myd88 and Irak4 induced NF-κB signaling.

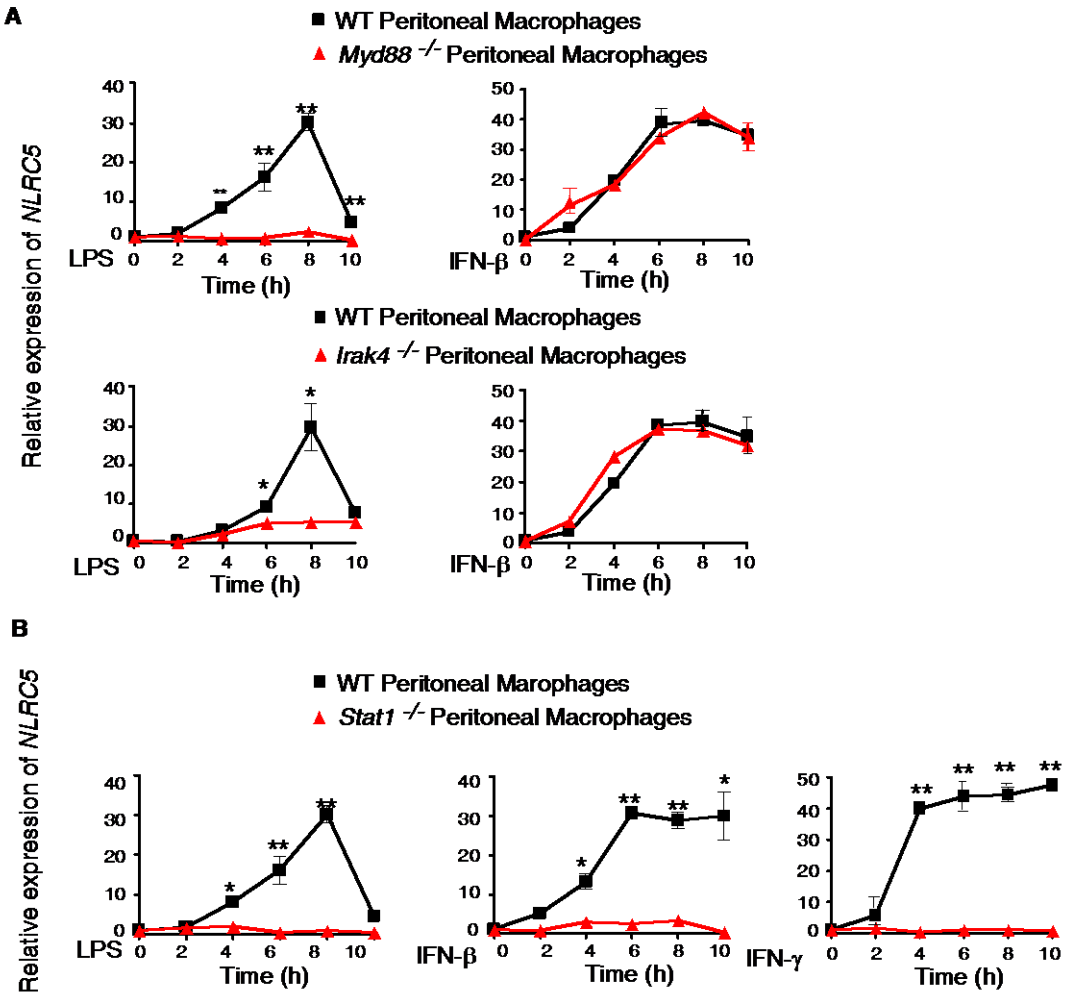


Figure 1. LPS induced NLRC5 in a Stat1-dependent manner. (**A**) Peritoneal macrophages isolated from WT, Myd88$^{-/-}$ or Irak4$^{-/-}$ mice were treated with LPS (100 ng/mL) or IFN-β (1000 U/mL). Total RNA was collected at indicated time points. The transcription level of NLRC5 mRNA was measured by real-time. (**B**) Peritoneal macrophages from WT or Stat1$^{-/-}$ mice were treated with LPS, IFN-β, or IFN-γ (10 ng/mL), total RNA at different time points was collected to analyze the NLRC5 relative expression by real-time PCR.

Stat pathway is activated IFNs and induce the expression of a number of genes that are important for anti-viral responses (O'Shea et al., 2002; Shuai and Liu, 2003)**.** LPS induced TLR4 signaling activates NF-κB transcription factor that regulate the expression of many pro-inflammatory cytokines, including IL-6, TNF-α, as well as interferons, such as IFN-α, IFN-β, and IFN-γ. Thus, we hypothesized that TLR ligands induce NLRC5 expression indirectly through the secretion of NF-κB activated cytokines. To exam our hypothesis, we stimulated $Stat1^{-/-}$ peritoneal macrophages with the reported reagents that are responsible for NLRC5 induction, including LPS, IFN-β or IFN-γ, and then NLRC5 expression was tested at different time points after stimulation. We found that Stat1 deficiency abolished the LPS, IFN-β, and IFN-γ induced the NLRC5 expression (Figure 1B). We concluded that LPS induced NLRC5 expression is regulated by transcription factor Stat1, whose activation is mediated by NF-κB activated cytokine release.

LPS indirectly induces NLRC5 expression through type I Interferon

To investigate if LPS induces NLRC5 expression directly through NF-κB pathway or indirectly through cytokines stimulating other pathways, we designed a supernatant transfer experiment to verify the role of released cytokines in gene transcription induction. We treated Raw264.7 cells with or without LPS for indicated hours, the cells were collected to analyze the expression level of NLRC5, and the supernatant were transferred to untreated Raw264.7 cells for an 2 hour incubation, and

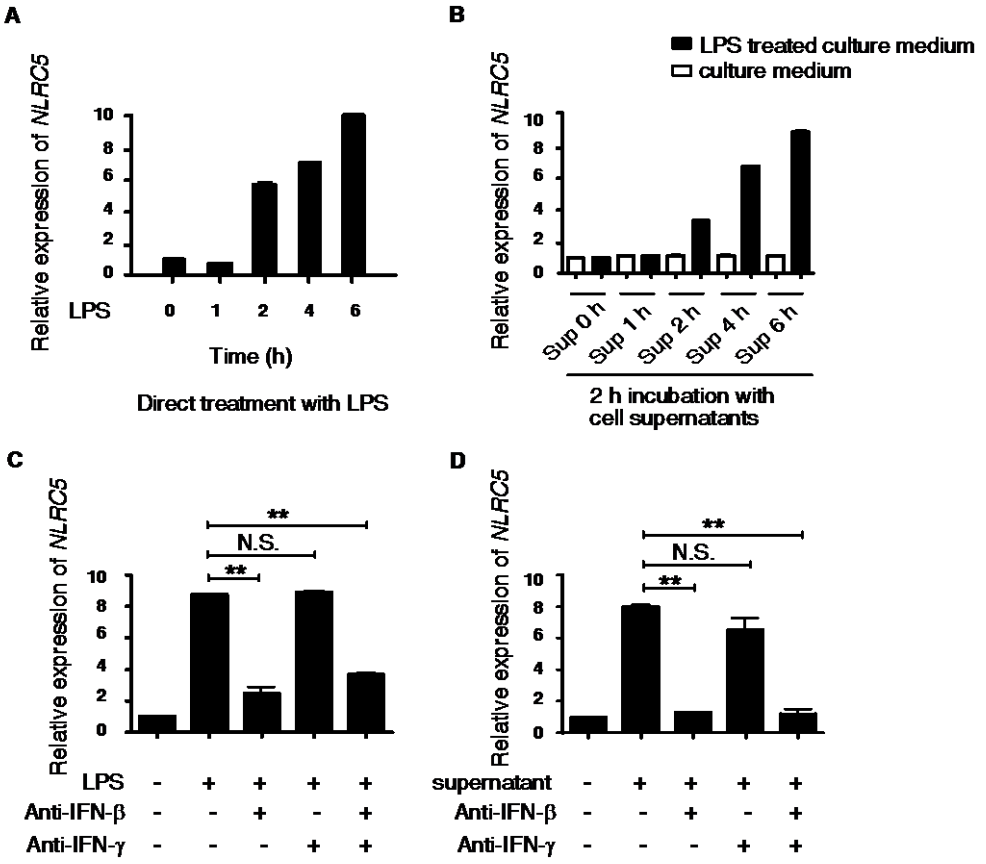


Figure 2. LPS-induced IFN-β release activates NLRC5 transcription. (**A**) RAW264.7 cells were treated with LPS (100 ng/mL) for 1h, and then the medium was replaced with fresh medium after wash. NLRC5 expression at different time points was analyzed. (**B**) Cell culture supernatants from the experiment in (**A**) were collected and immediately added to untreated RAW cells. NLRC5 expression after 2 h incubation with the supernatants was determined by qPCR. (**C**) RAW264.7 cells were incubated with medium, LPS, LPS+anti-IFN-β antibody, LPS+anti-IFN-γ antibody, or LPS+anti-IFN-β antibody +anti-IFN-γ antibody for 6 h, RNA were collected for NLRC5 expression analysis. (**D**) After 1 h of LPS treatment, RAW264.7 cells were washed to remove the LPS residue and then incubated with fresh medium for 5 h. The culture supernatant were collected and pre-treated with LPS+anti-IFN-β antibody, LPS+anti-IFN-γ antibody, or LPS+anti-IFN-β antibody +anti-IFN-γ antibody, and then transferred to unstimulated cells. RNA samples were collected after 2 h incubation for analysis of NLRC5 expression.

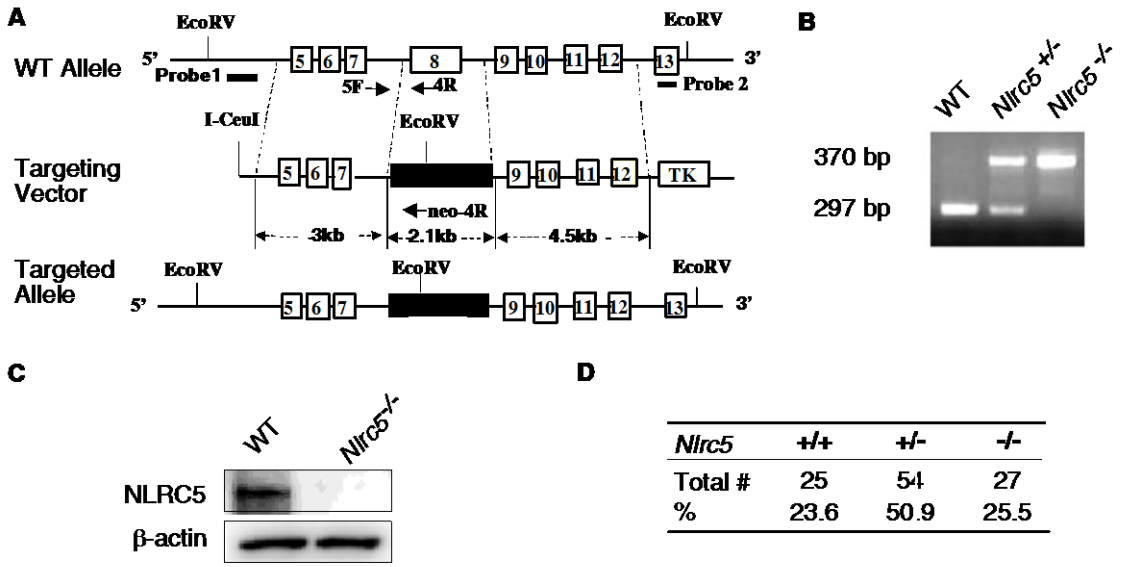
the supernatant treated cells were also collected to test NLRC5 expression. We found that NLRC5 mRNA was not only induced by direct LPS stimulation, but also induced by the treatment of supernatant from LPS treated cells (Figure 2A, B). We hypothesized that the molecules that released into the cell supernatant after LPS treatment led to NLRC5 induction. Upon TLR4-induced NF-κB signaling pathway activation and tye I IFN signaling pathway activation, several pro-inflammatory cytokines are released from the cytoplasm. As Stat pathway can be activated by type I or type II interferon(Guyer et al., 1995; Kovarik et al., 1998; Tassiulas et al., 2004), and its activation lead to the transcription of the regulated genes, we purchased neutralization antibody for IFN-β and IFN-γ so that the IFNs in the supernatant could be blocked. When the cells are treated with LPS with anti-IFN-β antibody, LPS induced expression of NLRC5 was abrogated; however, anti-IFN-γ antibody did not affect the LPS-induced expression of NLRC5 (Figure 2C). Similarly, we transferred the supernatant from LPS-treated cell culture to untreated cells, and found that anti-IFN-β antibody blocked the induction of NLRC5, but the anti-IFN-γ antibody did not (Figure 2D). In addition, anti-IFN-β antibody blocked almost all the induced expression of NLRC5 using the supernatant transfer protocol, indicating the IFN-β released by the cells after LPS stimulation plays the major role to induce the transcription of NLRC5. NLRC5 regulates type I IFN pathway as reported (Kuenzel et al., 2010), so it is important to find that the transcription of NLRC5 itself is regulated by type I interferon. The regulated induction of NLRC5 by IFNs forms a negative feedback loop to prevent excessive release of type I IFN and keeps a

homeostasis of the cellular microenvironment. The host would experience severe inflammation without the negative feedback mechanisms.

NLRC5 deficiency reduced MHC class I expressed by lymphocytes

Several groups provided evidences to support that NLRC5 has a positive regulatory function in MHC class I molecule expression. One group found that both the MHC class I mRNA transcription level and MHC I presenting molecule increased when NLRC5 was overexpressed (Meissner et al., 2010). They also found that NLRC5 may traffic to the nucleus and bind to the promoter region of the gene encoding MHC class I antigen so that NLRC5 positively modulated the transcription and presentation of MHC class I molecule.

We use NLRC5 deficient mice to verify the function of this gene in MHC I regulation, and to determine the physiological role of NLRC5 in NF-κB and type I IFN signaling regulation. Our group generated the NLRC5 deficiency mice by constructing a targeting vector replacing the exon 8 of NLRC5 coding region with a designed construct containing a sequence encodes protein resistant to neomycine (Figure 3A). The mice embryonic stem cells were transfected with the construct using electroporation. The chimeric mice were selected for breeding. After screening for the heterozygous of NLRC5 knockout gene, two heterozygous mice were set as breeding pair for NLRC5 homozygous knockout mice. The genotype of the mice were determined (Figure 3B). We also get the tissue from WT and NLRC5 KO mice to analyze the NLRC5 protein



Nlrc5	+/+	+/-	-/-
Total #	25	54	27
%	23.6	50.9	25.5

Figure 3. Generation of NLRC5 deficient mice. (**A**) Schematic of NLRC5$^{-/-}$ mice generation. (**B**) Genotyping using primers indicated in (**A**). (**C**) Detection for expression if NLRC5 protein in tissues isolated from WT or NLRC5$^{-/-}$ mice by western blot. (**D**) Mendelian distribution of the pubs bred by NLRC5 heterozygous mice.

level in the tissue, and we found that NLRC5 is completely not expressed in NLRC5 KO tissues (Figure 3D), showing the success of knocking out the gene. By observing the ratio of siblings of the breading pair, we found that the strain also follows the Mendelian ratio (Figure 3E).

To test the MHC I expression in mice lymphocytes, we need to isolate or purify the spleen or thymus cells for pure population of specific cell types. The most efficient way is to use cell staining and flow cytometry, so that the cell populations can be gated by the staining of the cell type-specific cell surface markers. Cells isolated from spleen, thymus or lymph node were stained with fluorescence antibody of CD4, CD8 or B220, which are namely the cell surface markers of $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes and B lymphocytes. Cells were also stained with H2-K^b antibody or I-A^b for antibody, here we used anti-H2-K^b for mouse MHC I detection and anti-I-A^b for mouse MHC II detection. We found that H2-k^b expression in $CD4^+$ T cells from NLRC5 deficient mice was significantly lower than in those from WT mice. Similar pattern were found in CD8 positive T cells that MHC I expression in these cells of KO mice dramatically decreased compared with that of WT mice (Figure 4A). Consistent with the observation in T cell populations, B cells in NLRC5 deficient mice also express decreased level of H2-k^b compared with cells from WT mice. We also check if NLRC5 deletion led to any effect on the MHC class II expression. Using the same method, we stained the cells with I-A^b antibody, and we found that not only the T lymphocytes, but also B lymphocytes isolated from $NLRC5^{-/-}$ mice expressed comparable level of MHC class II molecule, which is referred to I-A^b in this experiment, with those cells from WT mice (Figure 4B).

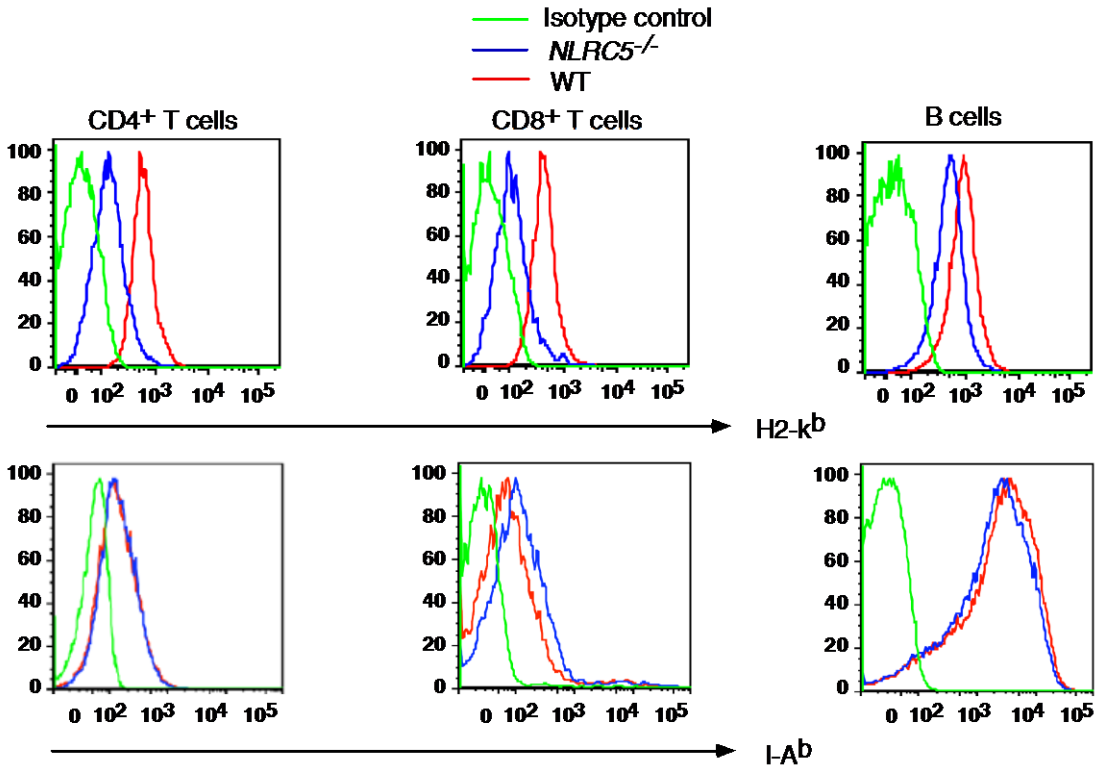


Figure 4. **NLRC5 deficiency decreased MHC class I presentation in lymphocytes.** H2-k^b (mouse MHC class I) and I-A^b (mouse MHC class II) staining in lymphocytes isolated from WT and NLRC5$^{-/-}$ mice were tested using flow cytometry.

Collectively, the observations indicated that NLRC5 positively regulates the transcription and presentation of MHC class I molecule in mice immune cells.

NLRC5 is an attenuator of TLR ligands induced NF-κB activation in mice embryonic fibroblasts (MEF)

Previous study found that NLRC5 interacts with IKKα/β, to reduce NF-κB activity in 293T cells and IKK α/β was observed to be released for IKK phosphorylation and increased NF-κB induced gene expression in Raw cells where NLRC5 is knockdown by siRNA (Cui et al., 2010). To validate NLRC5's role in NF-κB pathway in mice, we isolated MEFs from WT or NLRC5$^{-/-}$ mice, treated the MEFs with LPS, a TLR4 ligand, and then examined the post-trascriptional phosphorylation level of IKK and MAP kinases, such as p38, JNK and ERK. As shown in Figure 5A, phosphorylation of IKK in NLRC5 deficient MEFs was detected to be 3-fold stronger that in WT ones 15 minutes after LPS stimulation. However, activation levels of p38, JNK and ERK did not show significant difference between MEFs from WT and NLRC5$^{-/-}$ mice (Figure 5A). Besides the conditions resulted from NLRC5 deficiency which affected the NF-κB activation, we also tested whether NLRC5 deficiency led to increased expression of pro-inflammatory cytokines at the transcriptional level. We quantified transcription level of mRNA of IL-6, TNF-α, IL-1β, and IFN-β after LPS treatment using the total mRNA collected at different time points. The result showed that the transcription levels of IL-6, TNF-α, and IL-1β were 0.5 to 1 fold higher in NLRC5 deficient MEFs than those in WT

A

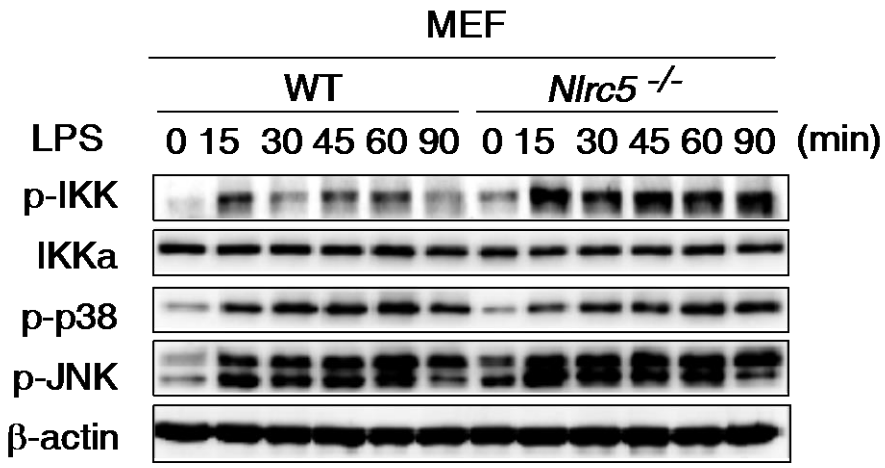
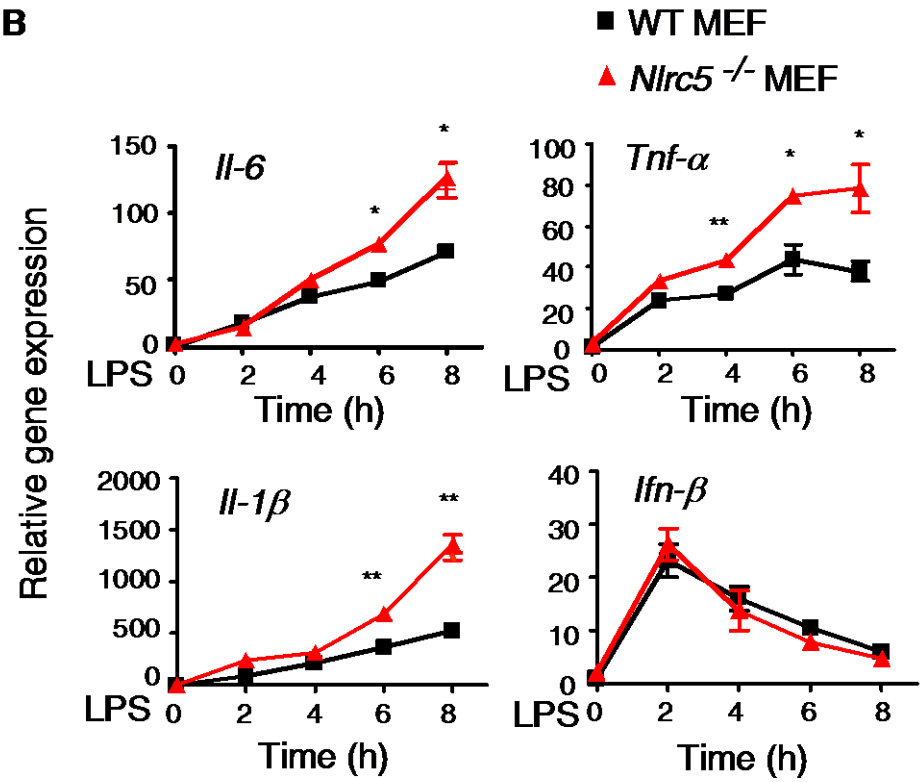


Figure 5. NLRC5 deficiency leads to enhanced NF-κB signaling after LPS stimulation in MEFs. (**A**, **B**) MEFs isolated from WT and NLRC5$^{-/-}$ mice were treated with LPS, and cell lysates collected as indicated for western blot (**A**) and total RNA was collected to determine the mRNA level of NF-κB induced genes by real-time PCR (**B**).



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ones 6 hours after LPS stimulation (Figure 5B). In contrast, the mRNA level of IFN-β, whose activation does not depend on IKK phosphorylation but on TLR4-TRIF-TRAM pathway, was normal in NLRC5 deficient MEFs. Herein, we concluded that knockout of NLRC5 enhances NF-κB activation and increases the production of NF-κB-induced pro-inflammatory cytokines in MEFs upon LPS stimulation, because of the lacking of NLRC5 to interact with IKKα/β and block the IKK phosphorylation.

NLRC5 deficiency increased TLR-induced pro-inflammatory response

When the innate immune system senses the pathogenic stimulation, the pro-inflammatory signaling is activated and leads to the expression and release of cytokines and chemokines which are dispensable components for inflammatory responses. The serious of innate immune responses induce more inflammation and recruit antigen presenting cells and other immune cells to defense the invasion. It would be important to know if NLRC5 knockout would have any effect on the cytokine release which directly affects the micro-environment for defending pathogens. To determine the effect of NLRC5 deficiency on the pro-inflammatory cytokine release after TLR stimulation, we treated the MEFs with ligands for different Toll like receptors, such as LPS, Poly(I:C), Cl097 and CpG, which are the ligands for activating TLR4, TLR3, TLR7, and TLR9 respectively. Also a time course treatment of different TLR ligands was carried out in MEFs, aiming to identify if the NLRC5 inhibits NF-κB signaling pathway through specific TLR pathway. We observed that the production of IL-6 and TNF-α in NLRC5

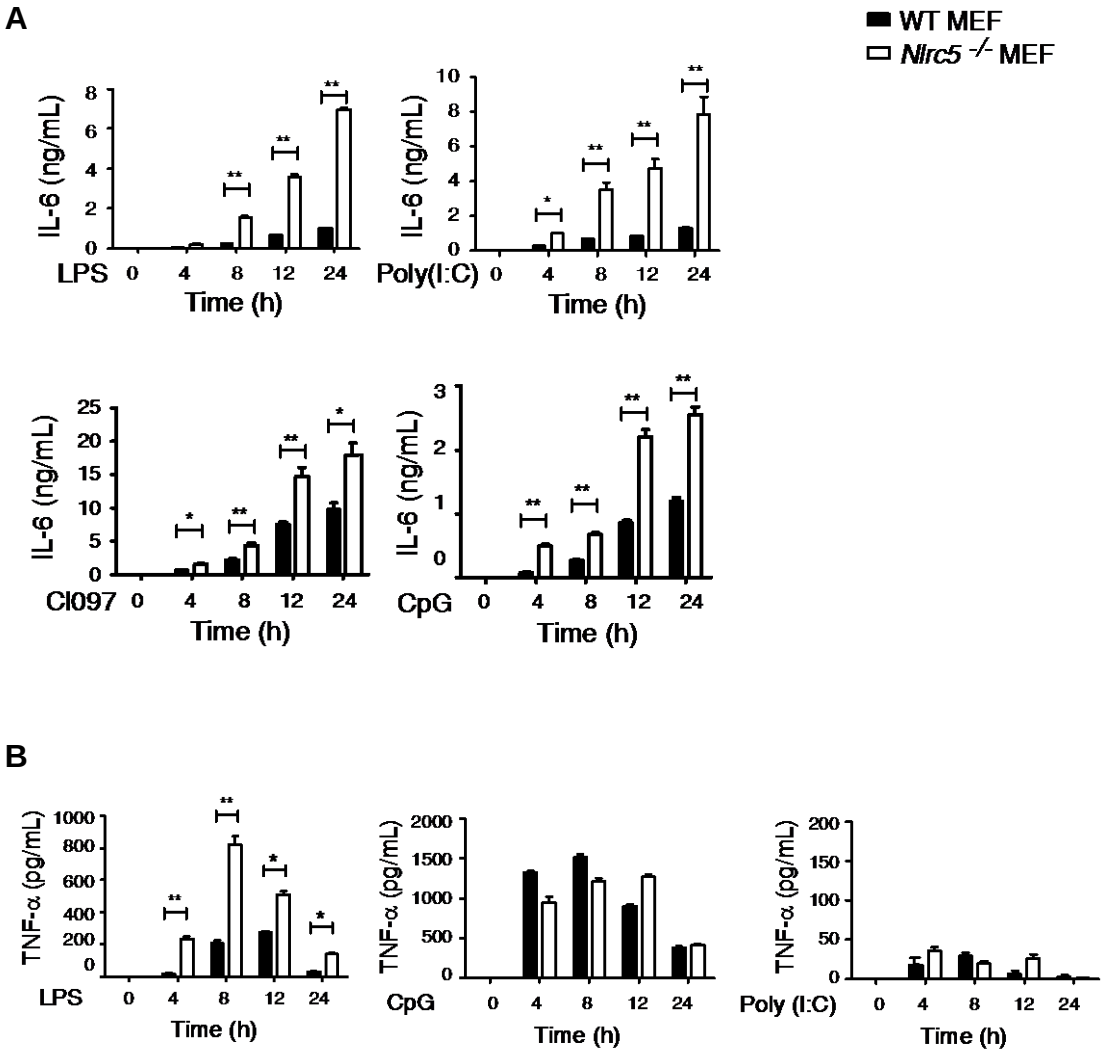


Figure 6. **Increased TLR ligand-induced production of pro-inflammatory cytokines in NLRC5-/- MEFs.** WT and NLRC5$^{-/-}$ MEFs were treated with different TLR ligands as indicated. The culture supernatants were analyzed for cytokine levels using ELISA.

deficient MEFs after different TLR ligand stimulations were all at least one fold higher than that of WT MEF cells at different time points (Figure 6). Especially, in the supernatant of NLRC5 deficient cells treated with LPS and Poly(I:C), we detected 3 to 5 fold higher level of IL-6 production compared to normal level. Thus, NLRC5 attenuates TLR-induced IKK phosphorylation and NF-κB regulated pro-inflammatory cytokine release. That is to say, NRLC5 functions as a important regulator to limit the secretion of pro-inflammatory cytokines and prevent severe inflammation that may lead to damage to the cells.

NLRC5 $^{-/-}$ embryonic fibroblasts are hyperactive in anti-viral responses to fight VSV

Anti-viral signaling or type I interferon signaling is another pathway that goes partially through TLRs. For examples, TLR can be stimulated by viral RNA or Poly(I:C) and activate type I interferon signaling. NLRC5 is able to interact with MAD5 or RIG-I to prevent the interaction with MAVS and block the activation of type I interferon signaling, which produces interferons to defend viral infection. It would be important to know if NLRC5 play a role in type I IFN pathway and regulate the anti-viral immunity *in vivo*. To exam if lacking of NLRC5 would affect the ability of immune responses to defend virus, we infected WT and NLRC5$^{-/-}$ MEF cells with VSV-eGFP and tested the type I interferon signaling at different time points. By detecting phosphorylated IRF3 using western blot, we found that phosphorylation of IRF3 occurred about four hours after the cells were infected with VSV. In addition, at about eight hours after the MEFs

were challenged with VSV, the IRF3 phosphorylation (quantified by measuring the band density) in NLRC5 deficient MEFs was 2-fold higher than that in WT MEFs (Figure 7A). We also analyzed the total IRF3 protein level in WT and NLRC5$^{-/-}$ MEFs, and found comparable expression level of IRF3 in both cell groups. Moreover, the total RNA of MEFs after VSV-eGFP challenge was collected at indicated time points after viral infection, and prepared for quantitative PCR so that we could determine the IRF3 activated transcription of type I IFNs and NF-κB induced genes of TNF-α and IL-6, which are induced by NF-κB activation. Correlated with the observation that NLRC5 deficiency increased IRF3 phosphorylation over time post viral infection, we revealed that expression of IFN-α and IFN-β in NLRC5 deficient MEFs was two folds higher than that in WT ones (Figure 7B). However, little or no differences of NF-κB induced TNF-α and IL-6 expression were detected (Figure 7B), and this result is consistent with the early observations in Raw 264.7 cells (Cui et al., 2010). In the aspect of cytokine release after rival infection, NLRC5 deficient MEFs released 1.5-fold higher of IFN-b protein after VSV-eGFP viral infection, when we measured the protein concentration in the cell culture supernatant by ELISA analysis. (Figure 7C), suggesting a negative role of NLRC5 in IRF3 phosphorylation and anti-viral cytokine production. Next, we treated the MEFs with poly(I:C)/Lyo vec treatment, that is also known to activate IRF-3 phophorylation. Similarly, significantly higher IFN-β release in the supernatant of NLRC5 deficient cells was revealed (Figure 7C). These results convinced us that NLRC5 inhibits type I IFN signaling by restricting MAVS-mediated IRF3 phosphorylation.

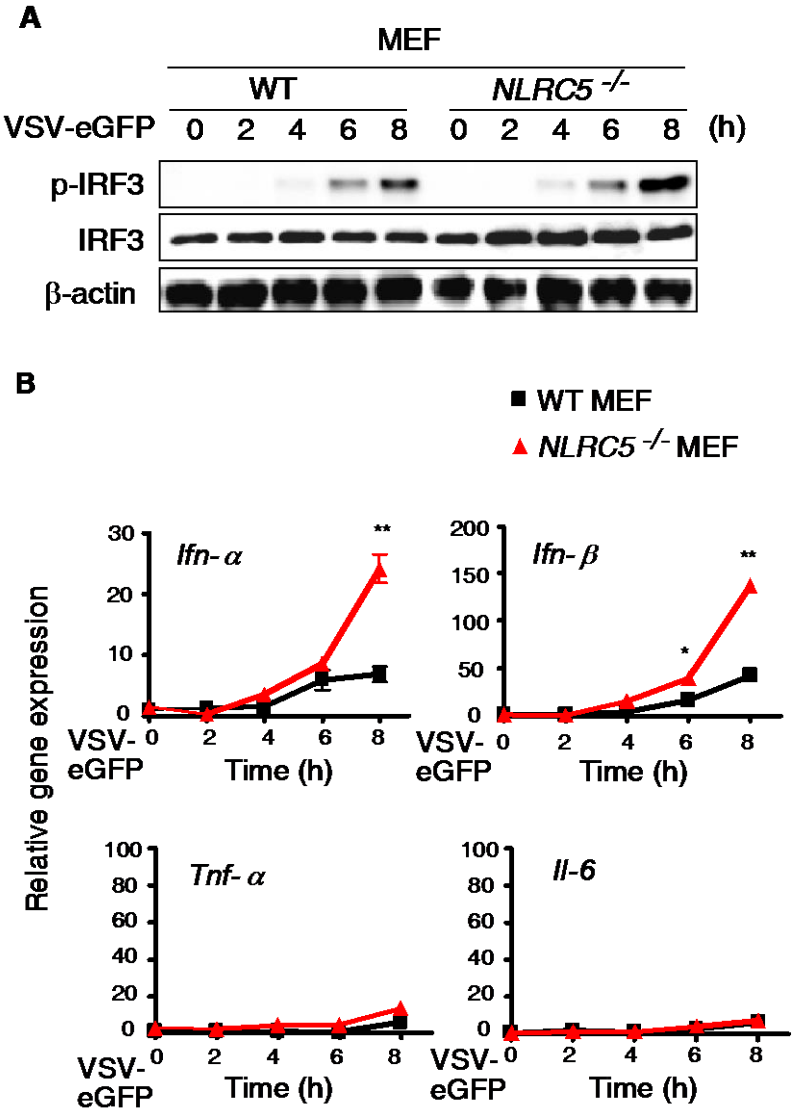
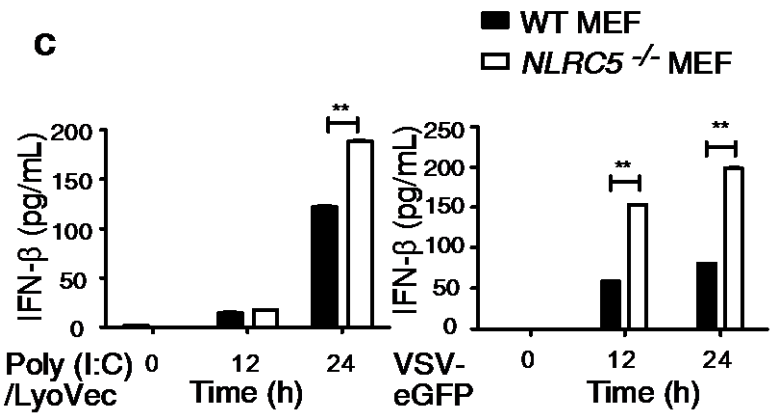


Figure 7. NLRC5 deficiency in MEFs increased IRF3-induced production of type I IFNs. (**A**) WT and NLRC5$^{-/-}$ MEFs were treated with VSV. Cell lysates at different time points after viral infection were collected with low salt lysis buffer to test IRF3 phosphorylation. (**B**) Total RNA of cells in (A) was harvested to exam the mRNA level of indicated genes using qPCR. (**C**) WT and NLRC5$^{-/-}$ MEF cells were stimulated with TLR3 ligands or VSV for the indicated time points. The production of IFN-β was analyzed by ELISA.



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NLRC5 deficient MEFs had stronger anti-viral responses than WT MEFs

The activation of type I interferon pathway after viral infection directly affects the anti-viral ability of the cells. Thus, it is essential to test the anti-viral responses in the NLRC5$^{-/-}$ and WT MEFs in response of viral challenge. By utilizing the GFP labeled VSV-eGFP for viral infection, we were able to quantify the infection or virus by observing the value of GFP fluorescence in the infected cells under fluorescence microscopy. NLRC5$^{-/-}$ and WT MEFs were challenged with GFP-labelled VSV and pictures under microscopy were taken at 24 hours after infection. WT MEFs showed stronger GFP fluorescence at 24 hours post infection, but NLRC5$^{-/-}$ MEFs did not show any GFP at the time point (Figure 8), revealing that NLRC5$^{-/-}$ MEF were merely infected by the virus at 24 hour after infection. Also, more of the WT MEFs were killed by the virus as showed in the phase microscopy (Figure 8). By showing that NLRC5$^{-/-}$ cells were much less permissible to viral replication (GFP fluorescence) and resisant to viral killing (Phase) comparing to WT ones , we linked the up-regulated IRF3-responsive cytokine release to the enhanced antiviral immunity in the NLRC5 deficient MEF cells. However, when we observed the cells at 36 h after infection, we found that NLRC5$^{-/-}$ and WT MEFs showed similar GFP fluorescence, and the dead cell numbers were comparable between these two groups. This result suggested that NLRC5 deficiency enhanced the anti-viral response in the early phase, but at the later time point, the immune response were compensated by other redundant genes that have the similar function as NLRC5.

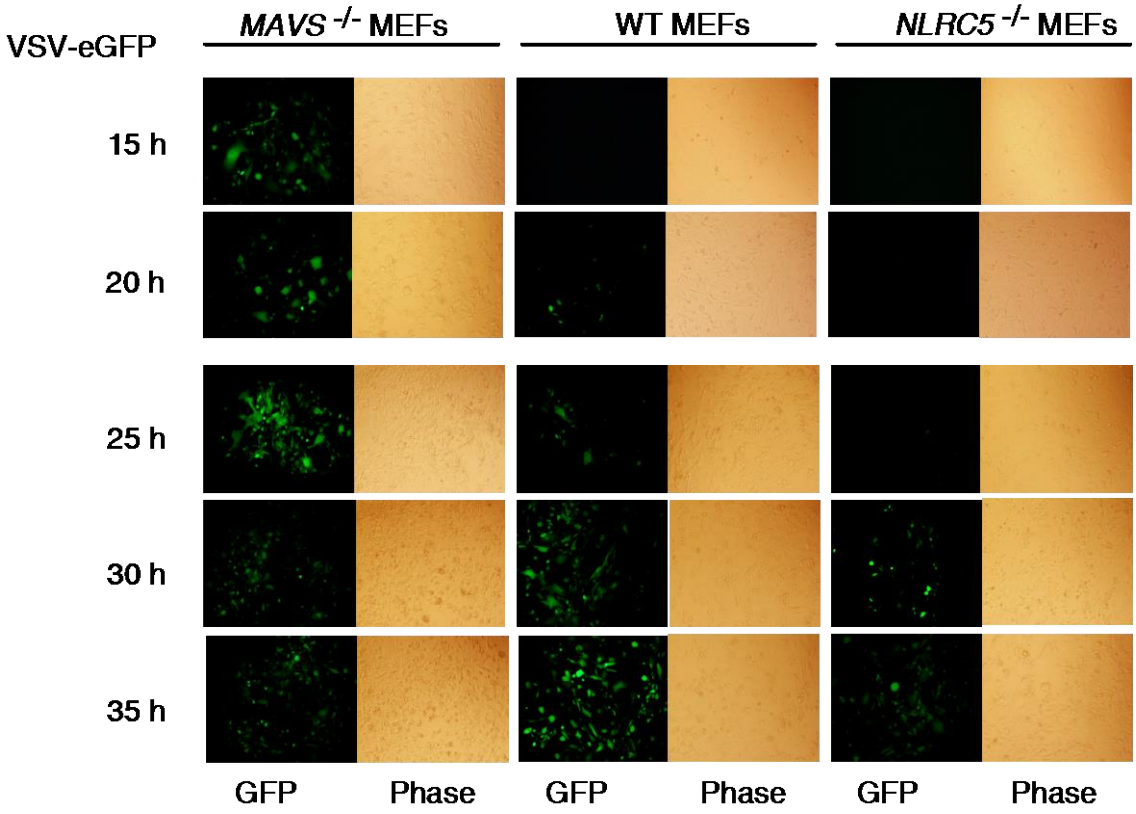


Figure 8. NLRC5 deficiency enhanced anti-viral responses in MEFs. WT and NLRC5$^{-/-}$ MEF cells were challenged with GFP-labelled VSV (MOI = 1), extent of viral infections or expansion indicated by GFP florescence were observed using fluorescence microscopy at indicated time points.

NLRC5 is a negative regulator of NF-κB and Type I IFN pathway in peritoneal macrophages

Even though MEFs express all molecules that are essential or indispensable for NF-κB and type I IFN signaling pathway, it is not physiologically functioning as immune cells. Thus, the analysis of immune cells from the NLRC5 deficient mice, such as macrophages and dendritic cells would be crucial for our study to reveal the physiological role of NLRC5 in innate immunity. We isolated peritoneal macrophages from the mice and treated these cells with LPS. As LPS stimulation not only activates NF-κB pathway through TLR4 signaling, but also activates MAPK signaling pathways and leads to the phosphorylation or activation of ERK, JNK, as well as P38, and these signaling pathways are activated as fast as in the first one hour after stimulation. Thus, we tested the phosphorylation of IKK and MAPK signaling molecules within the first hour after stimulation. IKK in the $NLRC5^{-/-}$ peritoneal macrophages was already activated without LPS stimulation, and the IKK phosphorylation was significantly increased in peritoneal macrophages isolated from NLRC5 deficient mice at 30 minutes and 60 minutes after LPS treatment (Figure 9A). However, MAPK signaling in WT and $NLRC5^{-/-}$ cells were activated in the same pattern. NF-κB signaling induced transcription of pro-inflammatory cytokines is another index for NF-κB activation. In WT peritoneal cells, IL-6 and TNF-α are induced by different patterns. IL-6 expression is activated 2 hour after LPS stimulation, and continues to increase until it

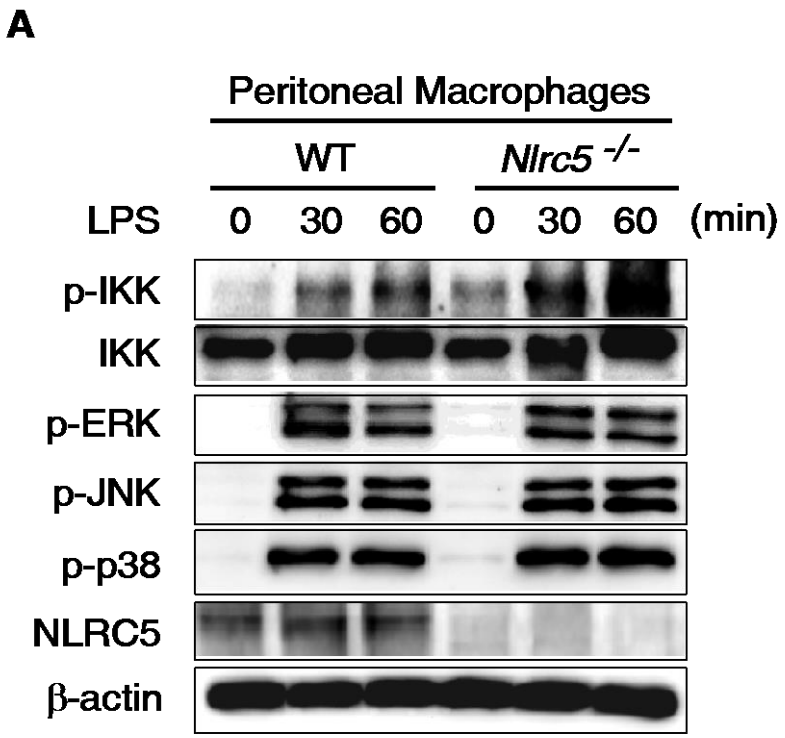
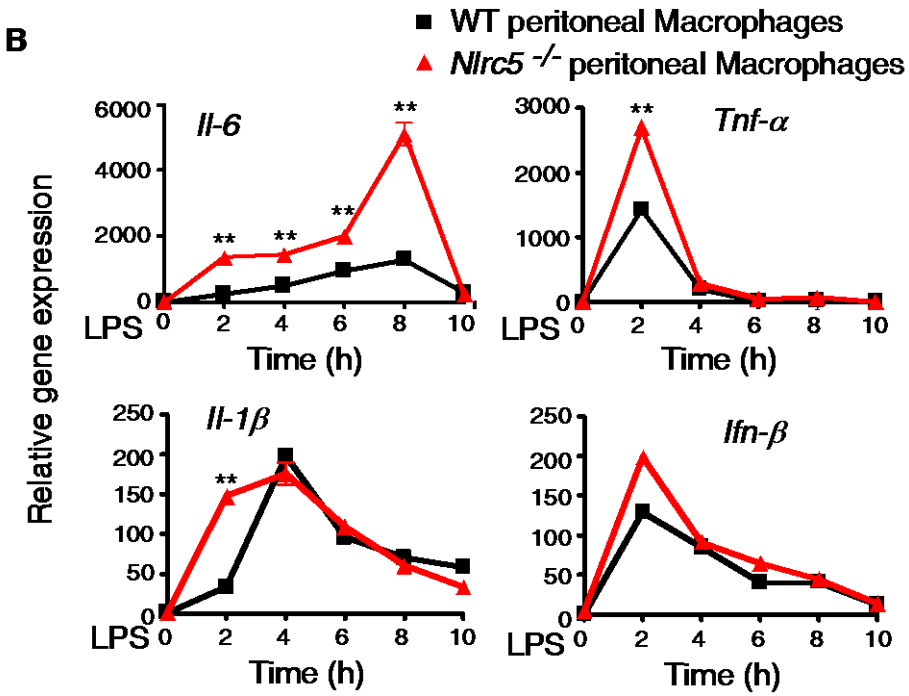


Figure 9. NLRC5 deficiency enhances NF-κB signaling after LPS treatment in peritoneal macrophages. (**A**) WT and NLRC5$^{-/-}$ peritoneal macrophages were examined for IKK phosphorylation and signaling activation after LPS (100 ng/ml) stimulation. Cells were lysed with low salt lysis buffer at different time points for western blot. (**B**) Total RNA was collected to analyze gene expression.



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reaches the peak at 8 hour after stimulation. TNF-α quickly reaches the highest mRNA expression at 2 h after LPS stimulation and then decreases reaches the peak at 8 hour after stimulation. TNF-α quickly reaches the highest mRNA expression at 2 h after LPS stimulation and then decreases (Figure 9B). Furthermore, NLRC5$^{-/-}$ peritoneal macrophages released higher level of IL-6 in response to LPS compared to WT ones, and expressed marked higher level of TNF-α, IL-1β, and IFN-β during early time points after LPS stimulation (Figure 9B). These result indicated that unlike the immune function in BMDCs, NLRC5 negatively regulates NF-κB in murine peritoneal macrophages.

NLRC5 deficiency enhanced TLR ligands induced cytokine release

Besides TLR4, other TLRs such as TLR3, TLR7, TLR9 can all activated NF-κB pathway. We would like to determine whether NLRC5 regulates only TLR4-induced pathway or ubiquitously regulates TLR-induced signaling. To validate the role of NLRC5 in pro-inflammatory response, we analyzed the cytokine production of peritoneal macrophage stimulated with multiple TLR agonists. WT and NLRC5$^{-/-}$ peritoneal macrophages both released IL-6 and TNF-α as early as 2 h after ligands stimulation. IL-6 production continued to increase until 24 h after stimulation. However, TNF-α production after LPS stimulation reached the peak at 6 hour after stimulation and decreased afterwards. We also observed marked higher release of IL-6 in response to LPS (TLR4 ligand), Cl097 (TLR7 ligand), and CpG (TLR9 ligand) stimulations in NLRC5$^{-/-}$ peritoneal macrophages compared to WT ones (Figure 10A). In contrast, we

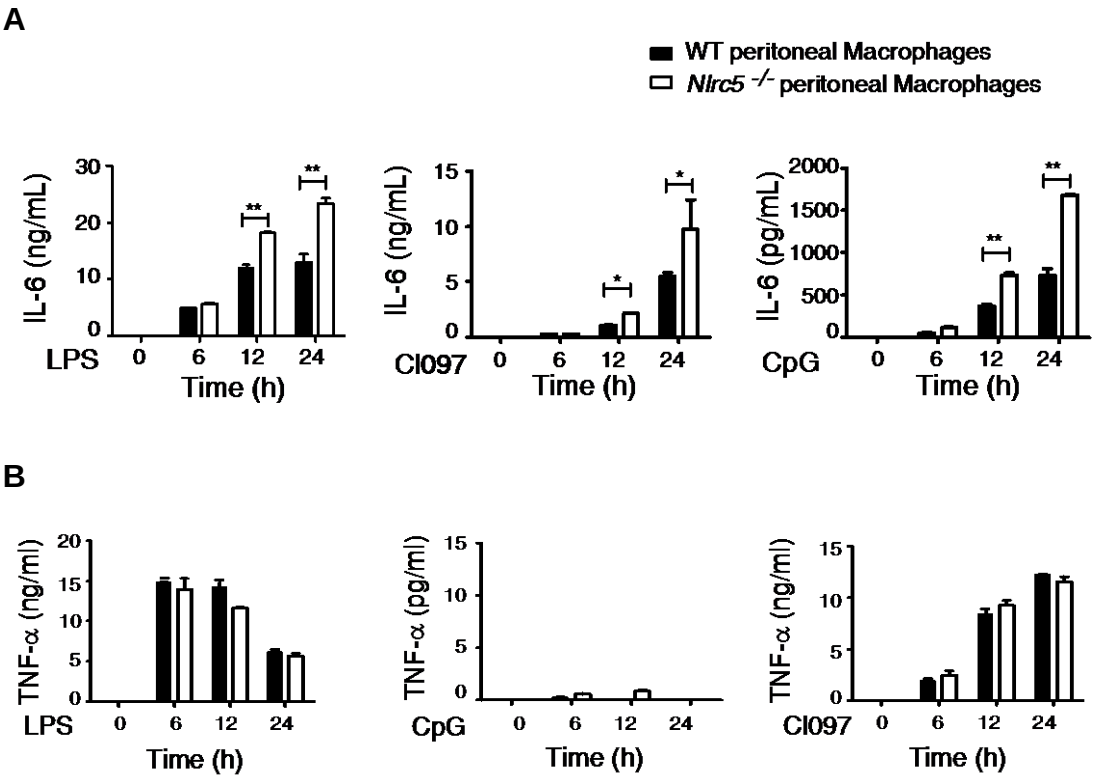


Figure 10. NLRC5 deficient peritoneal macrophages release higher IL-6 after TLR ligands stimulation. (**A, B**) Peritoneal macrophages were stimulated with multiple TLR ligands, and the cell culture supernatants collected at indicated time points were analyzed to determine the secretion of IL-6 and TNF-α.

did not find much difference in the TNF-α release between WT and NLRC5 deficient cells after stimulation with Toll like receptor ligands such as LPS, CL097 and CpG (Figure 10B). In summary, NLRC5 is a negative regulator in NF-κB signaling regulation and limits the pro-inflammatory cytokines production in peritoneal macrophages.

NLRC5 negatively regulates NF-κB pathway under a mild treatment of LPS

Even though NLRC5 deficiency significantly enhanced NF-κB activity and IL-6, TNF-α production in MEFs, it did not lead to large difference between peritoneal macrophages from WT mice and those from NLRC5 deficient mice, especially at later time points. We suspected that over-dose of treatment may result in saturation of the signals and vein the difference. To test this hypothesis, we treated the peritoneal macrophages with 10 ng/ml or 100 ng/ml of LPS. Cell supernatants after 6-hour incubation were collected to detect IL-6 and TNF-α release by ELISA. IL-6 and TNF-α production were both increased for more than 2 folds in the $NLRC5^{-/-}$ cells compared to WT cells when the cells were treated with lower dose (10 ng/ml) of LPS. However, when the cells were treated with higher dose (100 ng/ml) of LPS, we only found mild difference in IL-6 release, but not in TNF-α release (Figure 11). The results suggested that NLRC5 deficiency significantly enhanced pro-inflammatory cytokine release in peritoneal macrophages in response of mild stimulation of TLR ligand. It indicated that as a negative regulator, NLRC5 only affects the physiology response in the early time after treatment, and a lower dose of stimulations. NLRC5 may play a role to eliminate unnecessary signals for NF-κB activation to prevent auto-inflammatory diseases, and it

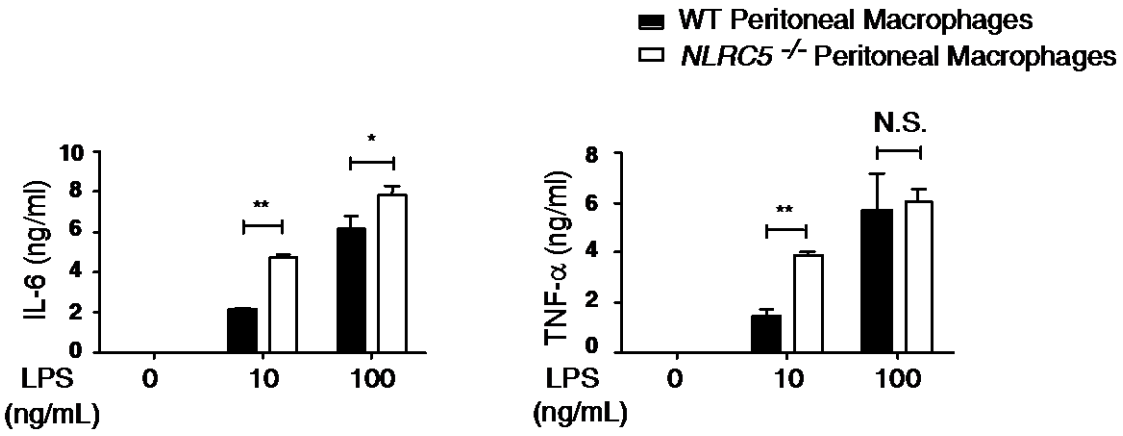


Figure 11. NLRC5 inhibits NF-κB signaling pathway under low dose of LPS in peritoneal macrophages. WT and NLRC5$^{-/-}$ peritoneal macrophages were treated with low dose (10 ng/ml) or high (100 ng/ml) dose of LPS for 6 h. IL-6 and TNF-α secretion in supernatants were analyzed by ELISA.

does not stop inflammation when the immune system need inflammatory response to defend pathogens.

NLRC5 deficiency enhanced the type I interferon signaling and anti-viral responses in peritoneal macrophages

We infected peritoneal macrophages with GFP labelled VSV to demonstrate the physiological function of NLRC5 in antiviral responses or type I IFN signaling pathway in these type of cells. Phosphorylation level of IRF3 initiated 4 h after viral infection in NLRC5 deficient cells. By contrast, IRF3 phosphorylation level in WT cells could not be detected as late as 8 h after VSV-eGFP infection. In addition, we detected increased IRF3 phosphorylation in NLRC5$^{-/-}$ peritoneal macrophages (Figure12A), which further supported the point that NLRC5 attenuates antiviral capability by preventing activity of antiviral response after virus infection in these cells. . And the GFP positive cells, which are cells infected by the VSV-eGFP were observed more in WT cells rather than in NLRC5$^{-/-}$ cells (Figure 12B), indicating that NLRC5$^{-/-}$ peritoneal macrophages have enhanced antiviral response activity compared to WT cells.

To elucidate that NLRC5 inhibit type I interferon pathway through RIG-I and MDA5, peritoneal macrophages were challenged with VSV-eGFP, Poly(I:C).Lyo Vec (intracellular dsRNA) that signal through RIG-I/MDA5), and TLR ligands (LPS, Poly(I:C), whose signaling is independent on RIG-I/MDA5. IFN-β release was

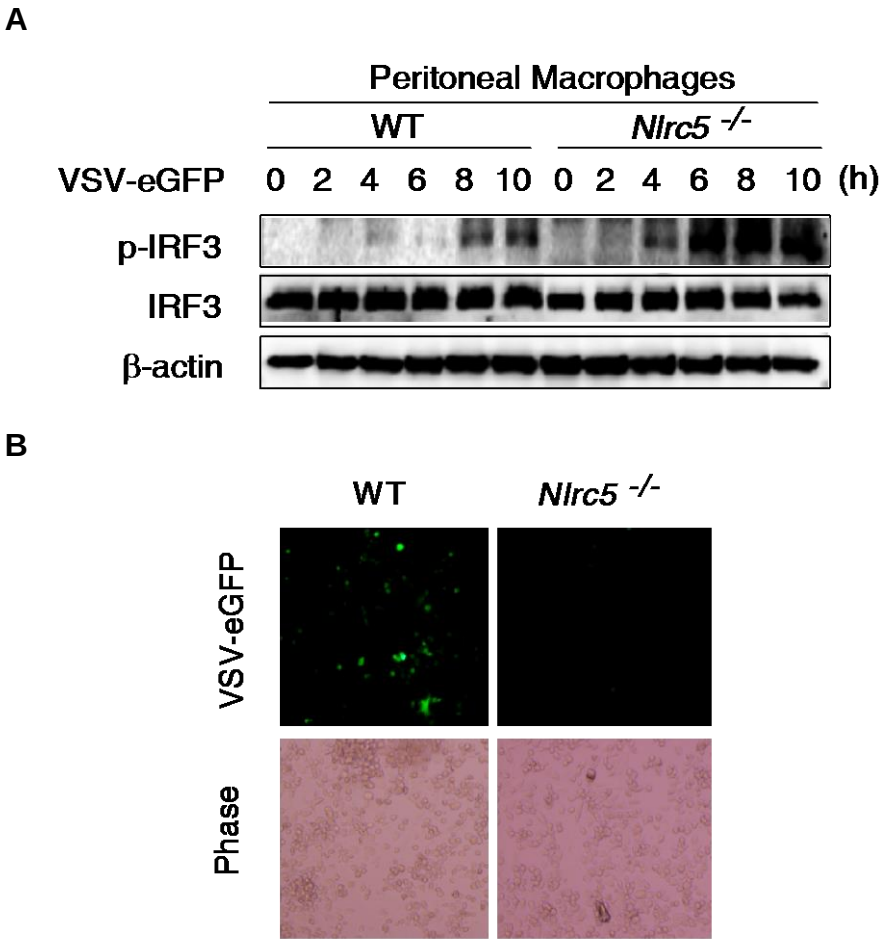


Figure 12. NLRC5 deficiency enhanced antiviral responses in peritoneal macrophages. (**A**) Peritoneal macrophages from WT and NLRC5$^{-/-}$ mice were infected with VSV-eGFP. Cell lysates at different time after infection were collected for western blot. (**B**) Cells were incubated with VSV-eGFP (MOI = 10), then extent of virus proliferation was observed at 24 h after infection using microscopy. (**C**) NLRC5$^{-/-}$ and WT peritoneal macrophages were stimulated with indicated ligands, and the culture supernatants at different time points were used to measure the IIFN-β production by ELISA.

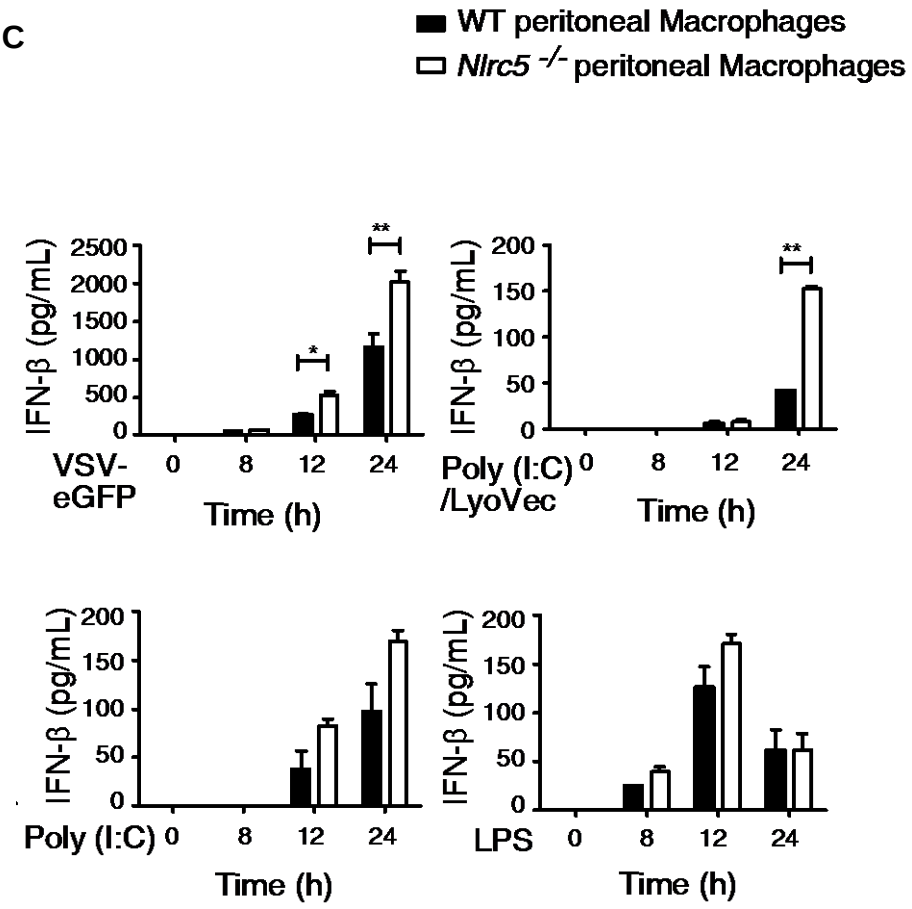


Figure 12. Continued.

significantly higher in cells from NLRC5 deficient mice in response to a type of dsRNA virus named VSV-eGFP or a ligand for RIG-I called intracellular Poly (I:C). However, IFN-β secretion level in the culture supernatant was normal when they were treated with ligands for TLR4 or TLR3, namely LPS or poly(I:C), which activates TLR4 or TLR3, respectively (Figure 12C). In conclusion, NLRC5 inhibits RLR-mediated type I interferon signaling pathway.

NLRC5 deficient peritoneal macrophages have normal inflammasome activity after stimulation with LPS and ATP

We observed that NLRC5 deficiency led to increased pro-IL-1β transcription after TLR ligands stimulation in both MEFs and peritoneal macrophages. NLRC5 is also reported to positively regulate NLRP3 inflammasome(Davis et al., 2011). According to the mechanism of NLRP3 inflammasome activation, pro-IL-1β need to be transcribed by NF-κB activation and then pro-IL-1β is cleaved by activated caspase-1 into mature IL-1β. Caspase-1 can be activated by various inducers, for instance, ATP can lead to the oligomerization of ASC and NLRP3 forming inflammasome and cleave caspase-1 into activated caspase-1 (P20). To determine the effect of NLRC5 deficiency in NLRP3 inflammasome activation, we treated the WT and NLRC5$^{-/-}$ peritoneal macrophages with LPS for 3 hours and then the cells were pulsed by ATP for 1 h. Cell supernatants were then collected to detect IL-1β release after stimulation. NLRC5$^{-/-}$ peritoneal macrophages released comparable level of IL-1β with the WT ones in response of LPS+

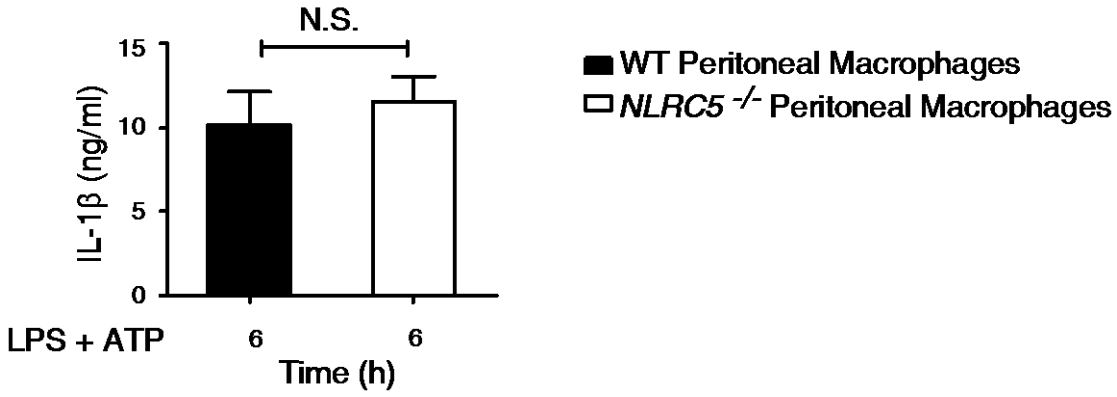


Figure 13. NLRC5 deficient peritoneal macrophages have normal inflammasome activity. WT and NLRC5$^{-/-}$ peritoneal macrophages were treated with LPS for 6 h and then with 0.5mM ATP for 1 h. IL-1β production was measured by ELISA.

ATP stimulation (Figure 13). These data revealed that NLRC5 deficiency did not influence the NLRP3 inflammasome activation in response of LPS+ATP.

NLRC5 deficiency pathway in bone marrow-derived macrophages enhances both NF-κB and Type I IFN signaling

To determine whether knockout of mouse NLRC5 expression also contributes to enhanced NF-κB signaling activation in other primary immune cells, we isolated bone marrow-derived macrophages (BMMs) and treated the cells with LPS. The result showed that the phosphorylation level of the TLR-induced signaling molecule IKK is markedly higher in BMMs from NLRC5 deficient mice comparing to those from WT mice (Figure 14A). However, the phosphorylation of JNK, ERK and P38 in the NLRC5$^{-/-}$ BMMs had no difference from that in WT BMMs (Figure 14A). Thus, LPS induced MAPK signaling pathways activation was comparable between NLRC5$^{-/-}$ and WT BMMs. When the BMMs from the mice were stimulated with LPS for different time points, IL-6 secretion from NLRC5$^{-/-}$ BMMs was significantly higher than that from WT ones (figure 14B). And we saw the most significant difference in the cytokine release at 6 hour and 12 hour after LPS stimulation, but not at 24 hour time point, suggesting that NLRC5 negatively regulates NF-κB pathway in early time points in response of TLR ligand stimulations. We also stimulate the BMMs with other TLR ligands, and found markedly increased release of IL-6 in NLRC5$^{-/-}$ BMMs after CpG or Cl097 stimulation at different time points (Figure 14B). We concluded that NLRC5 deficiency in BMMs significantly increased TLR ligands induced pro-inflammatory cytokine release.

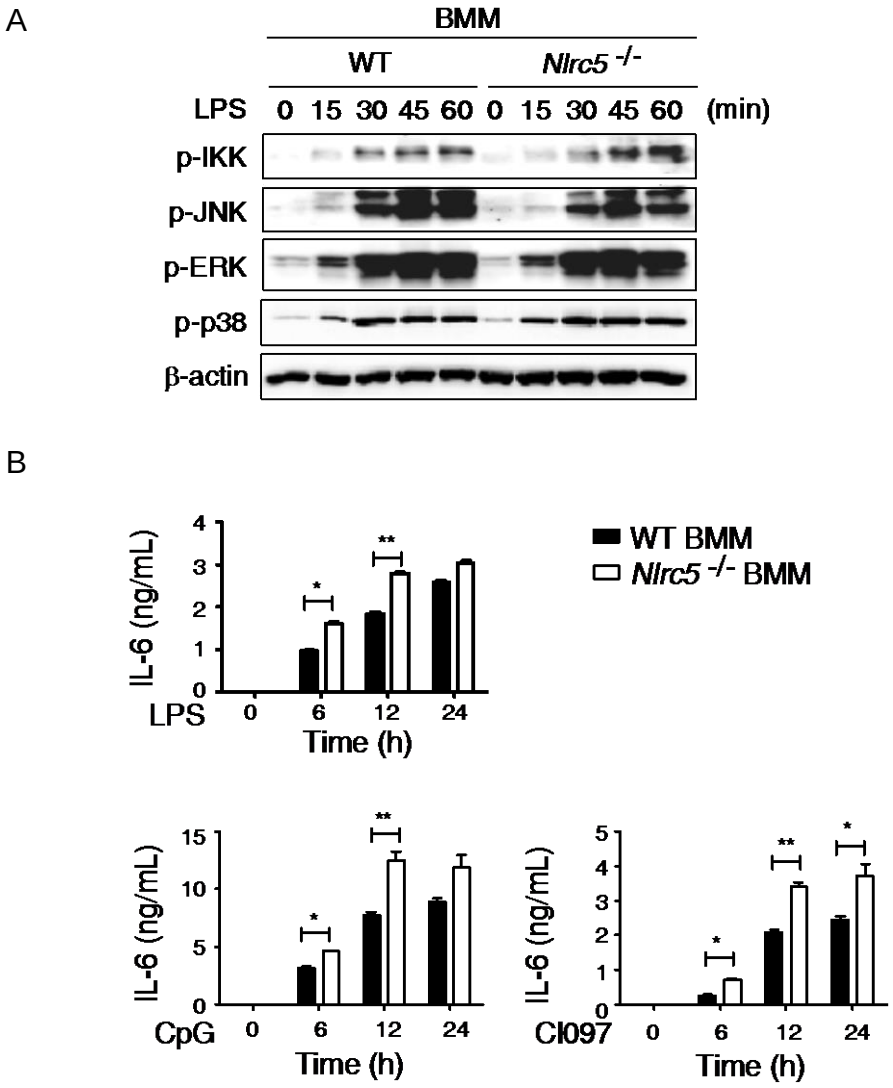


Figure 14. NLRC5 deficiency in BMMs increased TLR ligand-induced NF-κB signaling. (**A**) WT and NLRC5$^{-/-}$ BMMs were stimulated with TLR4 ligand LPS, and the cell lysates at indicated time points were prepared for western blot. (**B**) BMMs were treated with multiple TLR ligands such as LPS, CpG or CL-097, and the culture supernatants collected at indicated time were collected for ELISA analyses.

NLRC5 deficiency in BMMs increased type I IFN signaling

We also stimulated the BMMs with GFP-labelled VSV, so that the role of NLRC5 deficiency in dsRNA virus-induced anti-viral or type I IFN signaling of BMMs can be determined. Phosphorylation of IRF3 in BMMs was activated about 4 h after VSV infection in WT BMMs, and the phosphorylation accumulated upon time. We also detected increased phosphorylation level of the signaling molecule IRF3 at 6 h after the macrophages were infected with the virus in NLRC5$^{-/-}$ group (Figure 15A). Adding more evidence that NLRC5 inhibits antiviral responses, we evaluated the anti-viral response after infection with VSV-eGFP or transfected with Poly(I:C) by examining the viral infection-induced production of IFN-β by ELISA. NLRC5$^{-/-}$ BMMs secreted higher level of IFN-β compared to WT ones after stimulations activated through RIG-I/MDA5 pathway such as VSV-eGFP or Poly(I:C)/Lyo Vec at 24 h after VSV-eGFP infection, but not at 8h or 12 h post infection(Figure 15B). This time frame may depend on the process of viral infection, because we usually see GFP 24 hours after VSV-eGFP infection. Nevertheless, Poly(I:C) treatments did not make a difference for antiviral cytokine release between WT and NLRC5 KO BMMs (Figure 15B). Therefore, NLRC5 is also required for negative regulation of both TLR-induced NF-κB and viral infection or RIG-I/MAD5 mediated type I interferon pathway in bone marrow-derived macrophages. Herein, we concluded that NLRC5 deletion enhances not only NF-κB signaling pathway but also type I interferon pathway in macrophages.

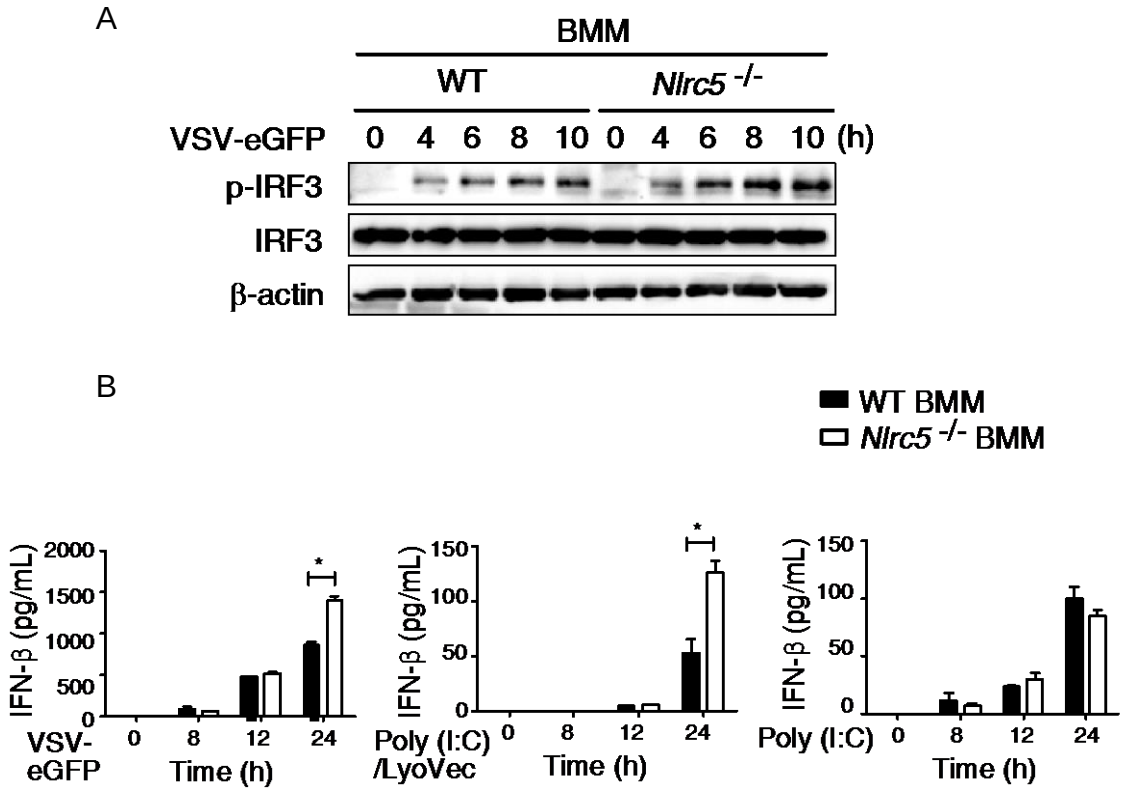


Figure 15. NLRC5 ablation in BMMs enhanced type I IFN signaling after viral infection. (**A**) WT and NLRC5$^{-/-}$ BMMs were challenged with GFP-labelled VSV. At indicated time points after infection as mentioned, the cell lysates were collected to test the phosphorylation level of signaling molecule IRF3 by western blot. (**B**) WT and NLRC5$^{-/-}$ BMMs were challenged with inducers of type I IFN pathway. ELISA was carried out using the culture supernatants to detect IFN-β production.

NLRC5 deficiency did not lead to difference in TLR ligands induced NF-κB signaling

Kumar's group using their NLRC5 knockout mice, tested NLRC5's function in NF-κB signaling in bone marrow derived or differentiated macrophages, and they found that NLRC5-/- and WT BMDC responded to the NF-κB activator with no differences (Kumar et al., 2011). In our study, we used the newly generated NLRC5 deficient mice that targeting the Exon 8, encoding the functional domain of the protein. We found that absence of NLRC5 led to stronger NF-κB signaling in response of multiple TLR ligands. We also generated BMDCs, and treated the cells with multiple categories of TLR agonists, such as LPS, CpG, Cl097, and Pam3scK. However, $NLRC5^{-/-}$ and WT BMDCs released comparable level of pro-inflammatory cytokine IL-6 and TNF-α. And we also tested IL-12 release, which did not show much difference between WT and $NLRC5^{-/-}$ cells as well (Figure 16). Thus, in BMDCs, NLRC5 does not play an important role in regulating NF-κB signaling in response of TLR signaling activation. This result may be explained by the cell type dependent manner, that specific type of cells achieves the signaling activation through different mechanisms. This would be further discussed in the discussion.

NLRC5 negatively regulates NF-κB and Type I IFN pathways *in vivo*

We next sought to determine the function of NLRC5 *in vivo* in LPS-induced septic shock. $NLRC5^{-/-}$ and WT mice were intraperitoneally injected with a high dose

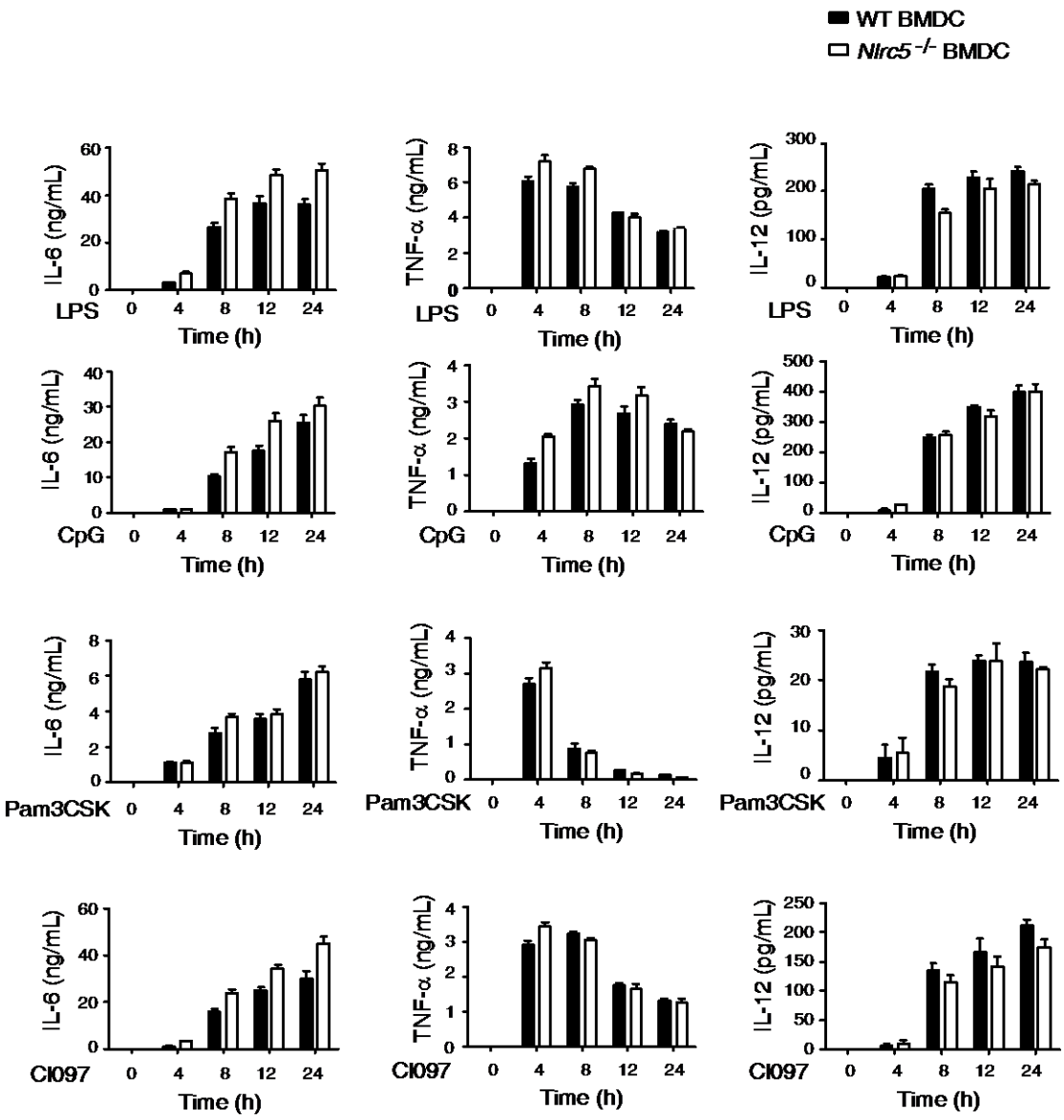


Figure 16. Normal TLR-induced cytokine release in *NLRC5*$^{-/-}$ BMDCs. WT and *Nlrc5*$^{-/-}$ BMDCs were treated with indicated TLR ligands for indicated times. IL-6, TNF-α, and IL-12 released in the supernatant were determined by ELISA.

of *Escherichia coli* LPS, and then the mice were monitored for their survival after septic challenge and the mice plasma were collected in early time points after LPS challenge. From the experiment, we observed that more than 80% of the NLRC5 deficient mice died within 48 hours, whereas 60% WT mice remained live (Figure17A). Even though there is a slight difference in survival between WT and KO group of mice, the difference is not statistically significant (Figure 17A). We also observed significantly higher serum IL-6 in NLRC5 KO mice (Figure 17B). Yet, this significant difference in plasma IL-6 level disappeared 3 h after LPS treatment. Moreover, the secretion of TNF-α in the plasma was comparable between NLRC5 deficient and WT mice (data not shown). These *in vivo* data give basis for the statement that NLRC5 negatively regulates NF-κB induced IL-6 production in the early phase during LPS-induced septic shock, however this effect fades as the treatment reached later phase.

NLRC5 deficiency did not make difference in anti-viral responses in response *of in vivo* viral infection

Even though NLRC5 deficiency enhanced type I IFN signaling and anti-viral responses in mouse fibroblasts and macrophages, it is still important to know whether the anti-viral ability of the mice would be affected in absent of the gene expression of NLRC5. To test whether NLRC5 knockout alternate survival after viral infection, we injected VSV-eGFP into NLRC5$^{-/-}$ and WT mice using tail vein (i.v) injection and then monitored their survival. Mice plasma was collected at different time points to inspect the viral infection and immune response reflecting by cytokine release in the plasma.

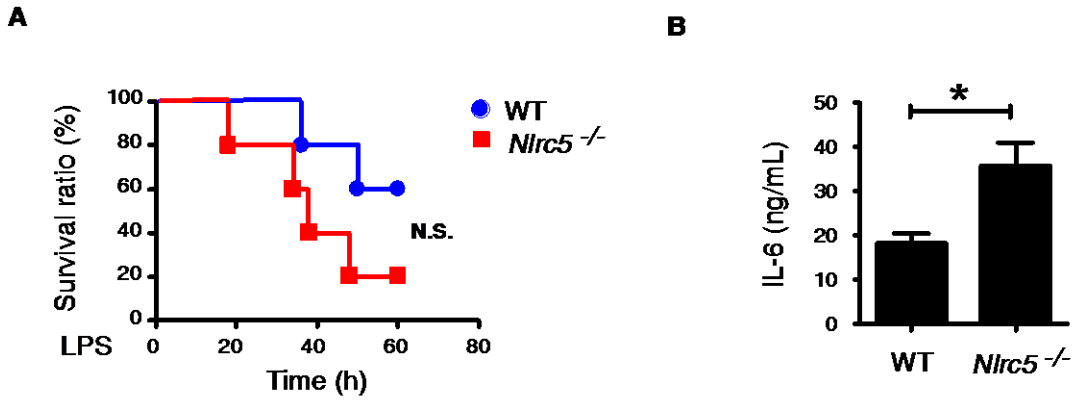


Figure 17. Functional role of NLRC5 in innate immunity under physiological conditions. (**A**) WT and NLRC5$^{-/-}$ mice, five mice for each group were injected with LPS, and the mice survival after challenge was observed. (**B**) Mouse sera were collected from WT and Nlrc5-/- mice when the mice were intraperitoneally injected with LPS for 1 h.

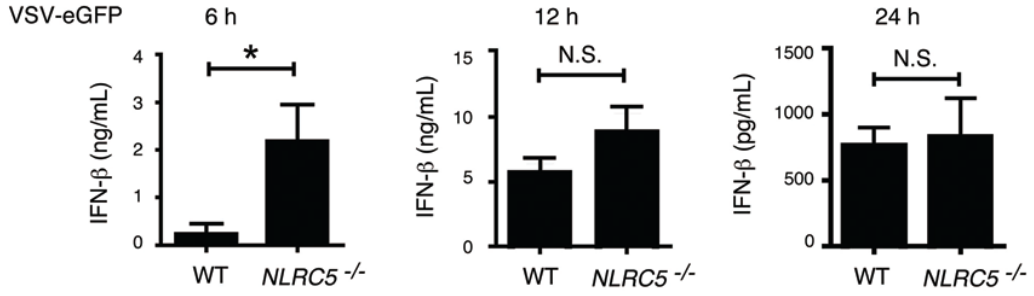
NLRC5$^{-/-}$ mice did not die under the low dose of virus infection as well as WT mice. We detected appreciable differences not in serum viral titers (Figure 18A, B), but in the IFN-β level released in to the plasma at 6 hour after VSV-eGFP infection (Figure 18A). In brief, NLRC5$^{-/-}$ mice release significantly higher level of anti-viral interferon at the early phase post viral infection. However, this difference did not sustain to the later phase of infection, and did not make difference in defending the viral invasion. Together, these results suggest that NLRC5 knockout promotes type I IFN pathway activity especially at a time frame when innate immunity is initiated, or relative early time in mice, but merely make difference for antiviral responses.

Discussion

Negative regulation of inflammatory pathways

Innate receptors (TLRs, NLRs and RLRs) activation triggers a cascade of signaling pathways (NF-κB activation, type I IFN activation) and leads to the production of various inflammatory cytokines such as IL-6, TNF-α and type I IFN, which have profound effects on the inflammation and tissue destruction. Plenty of evidence shows that dysregulation of these pro-inflammatory genes lead to unwanted or even fatal effect on the host by the over-activation of the innate immune system. In addition, such proinflammatory cytokine milieu has been shown to facilitate tumor development and progression. For example, production of IL-1 will lead to the differentiation of Th17 cells around tumors (Miyahara et al., 2008), and IL-17 in the tumor microenvironment

A



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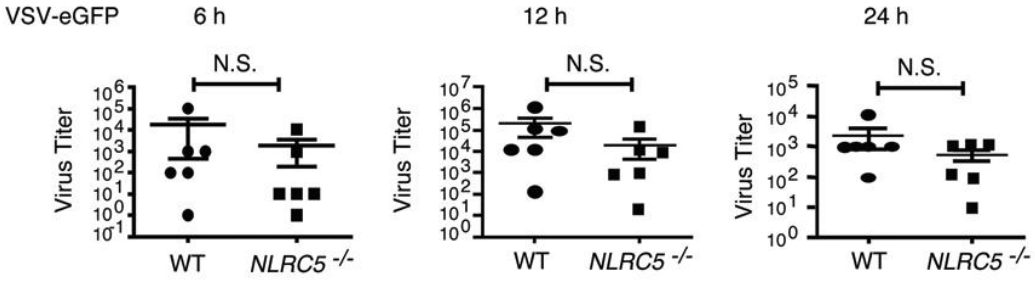


Figure 18. Increased type I IFN release in NLRC5$^{-/-}$ mice after viral infection. (**A**) IFN-β release in the serum of WT and NLRC5$^{-/-}$ mice at 6 h after they were challenged with VSV-eGFP using i.v. injection. (**B**) Serum collected from WT and NLRC5$^{-/-}$ mice at indicated time points after viral injection were used for virus titration.

produced by activation of Th17 cells can induce angiogenesis and help tumor cell survive(Chung et al., 2013).

NLRC5 in negative regulating NF-κB and anti-viral immunity

Many of the negative regulatory proteins themselves are transcriptional targets of NF-κB or IRFs, thus, a negative feedback loop is formed. NF-κB targeted A20, CYLD and DUBA are typical examples of negative feedback molecules. We initially observed the up-regulation of NLRC5 in both human cell lines and mouse primary macrophages is controlled by TLR4 induced NF-κB activity, suggesting a possible classical feedback loop. But there are possibilities that a secondary signaling pathway triggered by NF-κB induced products could be the real cause of induction of NLRC5. By promoter analysis, we and others found that NLRC5 promoter has a conserved STAT binding motif. It is possible that the STAT proteins induced by IFN-β/IFN-γ could induce the transcription of NLRC5. Both IFNs are induced by LPS induced NF-κB activation. By using STAT1 KO peritoneal macrophages, we found decisively that LPS induced NLRC5 expression was completely abolished in STAT1 KO cells indicating that the induction of NLRC5 observed in response of LPS stimulation is a secondary effect of IFNs activated by NF-κB pathway, though STAT1 dependent signaling pathway. The molecular mechanisms revealed provided another type of negative feedback type that the regulatory protein is induced by a secondary pathway triggered by NF-κB activation.

Previous studies indicate that NLRC5 can negatively regulate NF-κB signaling pathway and anti-virus pathway at IKK level and RIG-I level respectively(Cui et al.,

2010), but *in vivo* function of NLRC5 is not yet determined. Here, by generating NLRC5 KO mice, we found that NLRC5 regulates NF-κB activation and type I IFN production in a cell-type specific manner. We examined WT and KO cells include MEFs, peritoneal macrophages, BMDCs, BMMs, and we found that the most marked difference between WT and KO group is in MEFs. In macrophages, the difference is there but modest whereas in BMDCs, there is no difference between WT and KO group. The cell type specificity may due to several reasons: one is the mRNA transcription levels of the coding region of NLRC5 in these different tissues or different cell types vary. Indeed, the expression of NLRC5 differs dramatically among different cell types (Benko et al., 2010; Cui et al., 2010; Kuenzel et al., 2010). Another mechanism is that different cell types may utilize different molecular mechanisms for a particular pathway regulation. For example, TAK1 is shown to be a critical adaptor protein for NF-κB activation in MEF cells as well as in T cells and in B cells. However, one recent study found that TAK1 is not required but instead, exhibits negative effects in cell types such as neutrophils *in vivo*(Ajibade et al., 2012). Another example is TANK. Although it is reported to be an activator of IRF3 and NF-κB, it has been recently shown *in vivo* that TANK KO mice have enhanced NF-κB signaling by a possible *in vivo* role of promoting the ubiquitination and degradation of TRAF6 in response to TLR ligand binding(Kawagoe et al., 2009). Based on such observations, we contended that NLRC5 inhibits NF-κB signaling pathway induced by TLR ligands and type I IFN signaling induced by viral infection in MEFs and in a lesser extent in primary immune cells, especially macrophages, but not DCs. There are other groups developed NLRC5 KO

mice independently by targeting exon 4 and they reported no effect of NLRC5 in regulating inflammatory cytokines in macrophages and dendritic cells when the they are treated with TLR ligands or pathogens such as HSV-1, *L. monoctogenes* and NDV (Kumar et al., 2011). The experiments which are performed in BMDCs in our hand come out to be similar with their findings showing almost no difference between WT and NLRC5 KO cells. However, we truly found that in BMMs, there is significant difference after LPS or poly (I:C) challenge. The discrepancy may be resolved by three reasons. First, targeting different exons may have different outcomes in NLRC5 KO cells (exon 4 versus exon 8 deletion). Although mechanisms are not clear, there are examples of slight different phenotypes between KO mice with different exon targeting strategies such as SMAD3 KO mice generated by different groups. Secondly, a possible difference in the mouse background could also affect the outcome of *in vivo* experimental results. Although we backcrossed our NLRC5 KO mice with B6 background mice for more than 10 generations, it is still possible that traces of 129 background sequences in the genome will affect the experimental results. The third reason may due to different dose of LPS stimulation. In our hands, we use low dose of LPS whereas in other studies, high LPS dose (10 times higher compared to that we used) is used and may saturate the system. The saturated doses of LPS might overcome the effects of NLRC5 on the production of proinflammatory in WT and NLRC5-deficient cells. In addition, we found that ablation of NLRC5 showed more obvious difference in IL-6 mRNA trascription in both the cell types we looked at, namely MEFs and macrophages. However, the increase of another proinflammatory cytokine TNF-α is

much less obvious in macrophages. This might results from the multiple layers of TNF-α regulation. Cytokines such as pro-IL-1β and TNF-α are subjected to posttranscriptional regulation at different levels such as the mRNA stability and the protein translation(Han and Ulevitch, 2005). Such kind of discrepancy is common in immunology studies. Similarly, our group and others found that both NLRX1 knockdown or NLRX1 deletion led to dramatic change in the cytokine level of IL-6 but not another proinflammatory cytokine TNF-α, although the NF-κB activation mechanism is generally considered similar *in vitro*. NLRX1 knockdown leads to significantly higher IL-6 secretion level in the serum than the serum IL-6 detected in the WT mice after the mice were injected with LPS. However, the serum TNF-α level in the challenged NLRX1 knockdown mice was normal. It is possible that although the stimuli go through the common IKK-NF-κB complexes, the consequences of transcription of target genes may often be different with each other and more often than not are cell type specific, depending on some quantitative and qualitative properties of signaling in different cell types.

The functionally redundant negative regulators at the similar molecular level (IKK) such as CUEDC2 and NLRX1 in inhibiting NF-κB signaling may also contribute to the phenotype difference between *in vitro* and *in vivo* studies. *In vitro* studies often utilize knockdown assays and measure the cytokine immediately after gene knockdown by siRNAs or shRNAs. The cells barely have time to compensate for loss of one particular gene product. But *in vivo* gene deletion happens at the embryonic stage and the mice have plenty of time (6-8 weeks) to find a redundant molecular pathway to

compensate for the loss of one particular gene product. In this perspective, we generated NLRC5 KO and NLRX1 knockdown mice to study the possible compensation *in vivo*. Indeed, we found that in mice lack both molecules, the increase in pro-inflammatory cytokines such as IL-6 are significantly higher than single molecule deficient mice. This provides a new molecular mechanism of the possibility of compensation of functionally redundant genes *in vivo*. After all, there are still plenty of reports that cannot agree with each other in the field for unclear reasons. One example is that one group showed that NLRX1 KO mice exhibited increased level of IFN-β expression after infection with influenza virus(Allen et al., 2011), but other scientists observed NLRX1 does not have function in type I IFN signaling (Rebsamen et al., 2011). Thus, further studies have signaling to be performed to illustrate the exact role of one particular molecule *in vivo* before they can be applied to clinical studies.

In all, we showed that IFN-mediated Stat1 signaling is required to activate the transcription of NLRC5 mRNA when IFNs are sensed by the receptors. NLRC5 deficiency decreased the expression and presentation of MHC Class I genes majorly in T cells and in a lesser extent in other cells. NLRC5 deficiency enhanced LPS or VSV infection induced NF-κB signaling and type I IFN signaling pathways in a cell type dependent manner.

NLRC5 in MHC class I regulation

MHC class I molecules is comprised of a polymorphic heavy chain and a shared light chain (β2m). In human, heavy chains include HLA-A, HLA-B and HLA-C classes,

while in mice, heavy chains include H2-K, H2-D and H2-L classes. In human, the MHC class I heavy chain genes are located in a region of chromosome 6, where a number of severe diseases such as diabetes, asthma is associated to (Lie and Thorsby, 2005). Thus, MHC class I regulation must be very important for human diseases. Initially, studies found that one member of NLRC proteins which is called CIITA, when overexpressed *in vitro*, can activate the expression and presentation of MHC class I molecule expression (Martin et al., 1997; Williams et al., 2003). Due to the high homology of NLRC5 and CIITA as well as NLRC5 nuclear localization when overexpressed, it is intriguing to determine if NLRC5 can also regulate the expression of MHC class I gene. Indeed, it was reported that over-expression of WT NLRC5 can induce MHC class I genes such as HLA-A, HLA-B, HLA-C, HLA-E, HLA-F, HLA-G, β2m expression as well as genes responsible for MHC class I processing and presentation such as TAP1 and LMP2 (Meissner et al., 2010), however, the NLRC5 mutant lacking the NLS sequence failed to do so. The up-regulation of MHC molecules is restricted to MHCI instead of MHCII indicating that NLRC5 specifically activates MHC class I genes. *In vitro* siRNA knockdown of NLRC5 assays also confirmed the function (Meissner et al., 2010). But the *in vivo* evidence of NLRC5 regulating MHC I molecules is still lacking. The fact that CIITA deficient mice do not have defects in expression of MHCI indicating a possible *in vitro* artifacts. By generating NLRC5 knockout mice via replacing exon 8 in the cDNA of NLRC5 gene, we used FACS analysis to determine MHC class I presentation on the surface mouse lymphocytes. We found that NLRC5 positively regulates MHC class I expression because we observed that in NLRC5 knockout mice, the expression or

presentation level of H2-K (B6 background) is defective in many cell types including CD4+ T cells, CD8+ T cells, B cells, DCs, macrophages and neutrophils. The biggest difference of expression is in T cells whereas the difference is milder in cDCs, B cells, macrophages and neutrophils. This result indicated that NLRC5 specifically regulate MHC class I expression *in vivo* in a cell type specific manner. However, the expression of MHC class II molecules were comparable between WT and knockout group in all the cell types examined. In the meantime, an independent study using NLRC5$^{-/-}$ mice obtained through targeting the exon 4 on cDNA of the coding region for NLRC5 confirmed our observation *in vivo* by showing NLRC5 deficient T, NKT, NK and γδT cells have dramatically impaired MHC class I molecule expression and are also defective to be induced by IFN-γ (Biswas et al., 2012; Robbins et al., 2012; Staehli et al., 2012). Because MHC class I complex is very critical for the recognition of antigens by CD8+ T cells, the defective MHC class I expression in the NLRC5 deficient mice could lead to a defective CD8 T cells response. Consistent with this notion, the CD8+ T cell activation were indeed reduced in NLRC5 KO mice when challenged with specific antigen (Yao et al., 2012) and was not able to clear the intracellular pathogen *L. monocytogenes in vivo*(Staehli et al., 2012; Yao et al., 2012). Because tumor cells are often down-regulating MHC class I to avoid immune surveillance, it is possible that NLRC5 down-regulation is utilized by tumor cells. Firstly, it is found to be true that the expression of NLRC5 is very low in tumor cell lines related to the dysregulation of T and B cells (Staehli et al., 2012), indicating certain types of tumors are using such mechanisms to avoid being attacked by CD8+ T cells. We are looking for tumors which

have NLRC5 mutations in tumor cells by sequencing exons of NLRC5 in tumor cells. Until now, only very few mutations were found in NLRC5 coding regions in tumor cells. The future work should increase the sample size and also perform regulatory regions of NLRC5 sequencing given the fact that the sequencing technologies are growing super-fast during these years. It will be important to investigate whether NLRC5-MHCI axis is associated with anti-tumor immunity, anti-viral immunity and anti-allograft transplantation.

CHAPTER III

FUNCTIONAL CHARACTERIZATION OF DDX46 IN NLRP3 INFLAMMASOME ACTIVATION

Introduction

Inflammasome

The concept of inflammasome was first introduced by Tschopp and his colleagues in 2002(Martinon et al., 2002). Inflammasome is a huge protein complex that forms in the cytoplasm to serve as a platform to recruit and activate pro-caspase-1. Among the caspases, caspase-1 activation is the most well characterized in inflammasome activation and the consequence of caspase-1 activation is proteolytic cleavage of pro-IL-1β and pro-IL-18 and release of mature cytokine into the extracellular environment(Cerretti et al., 1992; Thornberry et al., 1992). It has become clear now that there are distinct inflammasomes which are scaffolded by different proteins, mainly by members of NLR family members containing nucleotide-binding domain (NBD) and leucin-rich repeats (LRRs). The characterized inflammsome includes: NLRP1, NLRP2, NLRP3, NLRP4, NLRP5, NLRP6, NLRP9, NLRP10, NLRP12, NLRP14, NLRC4, AIM2 inflammasomes.

NLRP3 inflammasome

NLRP3 was first reported in 2002 that it activates caspase-1, and later described to be responsible for autoinflammatory periodic fevers, for example, Muck-Wells syndrome(Martinon et al., 2009; Strowig et al., 2012). Unlike NLRP1, NLRC4 inflammasome, NLRP3 is not constitutively expressed in most unstimulated cells. NLRP3 expression can be induced by PAMP detection through TLR activation and NF-κB signaling pathway. However, NLRP3 activation requires a second signal to induce oligomerization. These secondary stimuli include crystalline stimulations (alum, uric acid crystals, silica) (Dostert et al., 2008; Eisenbarth et al., 2008; Martinon et al., 2006), ATP (Mariathasan et al., 2006), viral infections (Ichinohe et al., 2009), fungal products (Joly and Sutterwala, 2010), bacterial nucleotides (Kanneganti et al., 2006; Muruve et al., 2008; Sander et al., 2011) and numerous toxins (Vance et al., 2009). Since there are so many inducers for NLRP3 inflammasome activation, it is not clear how a single molecular mechanism can unify all the different ligands to activate the inflammasome. After all, these stimulations usually lead to calcium or potassium efflux, ROS change. There is a big unsolved problem that how NLRP3 is activated by these DAMP signals. In general, stimuli can be divided into two categories, crystalline and noncrystalline. For crystalline stimuli, the common mechanism is based on the lysosomal destabilization that results in release of enzymes such as cathepsin B and in turn activates NLRP3(Hornung et al., 2008). For noncrystalline NLRP3 activation, GBP5 is required for optimal NLRP3 activation and oligomerization (Shenoy et al., 2012). Another model for NLRP3 activation proposes that reactive oxygen specis (ROS) generated by

NADPH-oxidase during phagocytosis or by mitochondria can dissociate TXNIP from thioredoxin and TXNIP can be a ligand for NLRP3(Zhou et al., 2010). But models regarding ROS hypothesis remains highly controversial by Txnip KO or Panx1 KO studies showing normal NLRP3 activation in knockout mice(Masters et al., 2010; Qu et al., 2011). But in all cases, the exact detailed molecular mechanism is still lacking. The post-translational modification of NLRP3 is related to its activation. Ubiquitination of inflammasome complex lead to inflammasome degradation, accordingly, deubiquitination of NLRP3 would prevent inflammasome from degradation, so that inflammasome is activated. So far, the only reported post-translational modification of NLRP3 is deubiquitination by BRCC3(Py et al., 2013).

DDX/DHX family members and DNA/RNA sensors

Equal effort has been focused on finding the activating sensor of inflammasome in response of different inflammasome stimulations. DHX33 has been found to directly bind to double strand RNA and activate NLRP3 being an example of sensors that might detect different categories of PAMPs and DAMPs to activate NLRP3 inflammasome (Mitoma et al., 2013). Thus, it is probable that other members of the DDX and DHX gene family members can be sensors of different kinds of nucleic acid because they have conserved helicase domain sequences to serve as RNA helicases. Until now, there are 36 members of DEAD family and 14 members of the DEAH family to be putative nucleic acid helicases. The DHX members are involved in pre-mRNA splicing such as DHX15, DHX16 and DHX38. It is interesting that some DDX members are dysregulated in

cancer involving in chromosomal translocations. And consistently, some DNA helicase mutations are found to be important increasing the incidence of cancer types (Van Brabant et al., 2000). But the functions of DDX and DHX family members in immunity are just being studied. Due to their nucleic acid binding ability, DDX and DHX family proteins are potential DNA/RNA sensors in the cytoplasm and provide specificity to different stimuli. Actually, RIG-I (DDX58) and LGP2 (DHX58) both belong to the family and are famous RNA sensors in type I IFN pathways (Beutler et al., 2007). And by biochemical purification of CpG DNA binding proteins, DHX36 and DHX9 are found to be sensors of CpGA and CpGB in pDC cells (Kim et al., 2010). Here, by screening inflammasome activation in 293-ASC-caspase-1-IL-1β cells, we found that DDX46 is involved in inflammasome activation. By ligand screening, we further identified that DDX46 can be cleaved upon crystalline stimulation and the cleaved form of DDX46 can significantly bridge the interaction of NLRP3 and ASC to activate caspase-1, leading to optimal NLRP3 inflammasome activation and IL-1β production *in vitro*.

Materials and Methods

Cell lines

293T-caspase-1-ASC cell line has been described previously (Yu et al., 2007; Yu et al., 2006). Stable 293T-caspase-1-ASC-pro-IL1β cell line was obtained by transfecting 293T-caspase-1-ASC with expression plasmid pMSCVgfp-pro-IL1β. Stable

cell lines were generated after multiple cell-sorting by flow cytometry over a period of one month. 293T-caspase-1-ASC-pro-IL-1β-NLRP3 cells were generated by infecting the 293T-caspase-1-ASC-pro-IL1β cells with lentivirus expressing NLRP3. Expression of NLRP3 of the constructed cell line was verified by western blot, and the inflammasome activation system utilizing this cell line was tested and proved to be effective for inflammasome activation.

ELISA for human IL-1β

Put all samples and reagents to room temperature. Make sure to duplicate each samples and standard curve. Prepare standard dilutions and samples using sample diluents as indicated by manufacturer. Get the pre-coated plate strips as needed. Add 200 µL of Standard dilution, control or sample to each well. Cover the strip with a plate sealer, and incubate at room temperature for 2 hours. Wash the plate for 3 times. Then add 200 µL of Conjugate to each well. For samples of cell culture supernatant: cover the plate with a new plate sealer, and incubate the plate at room temperature for 1 hour. Aspirate and wash 3 times. Add 200 µL of substrate solution to each well. Incubate the plate at room temperature for 20 minutes in dark. Finally, add 50 µL of stop buffer to each well. Read at 450 nm with a plate reader within 30 minutes. Set wavelength correction to 570 nm.

Protein precipitation of cell culture supernatants

Cells were grown in non-serum medium with specific ligands as indicated, cell culture supernatants were collected at indicated time points. Proteins in the cell culture supernatants were precipitated with an equal volume of methanol and 0.25 volume of chloroform. Then the samples were vortexed and centrifuged at 16000g for 15 min. The upper phase was discarded and the precipitate was washed with one volume of methanol followed by centrifugation for 15 min at 16,000g. The upper phase was discarded. The protein pellet was dried in 55 ℃ for 10 min. Then the pellet was resuspended in 4xSDS protein loading buffer and boiled for 5 min at 100 ℃. The presence of caspase-1 and IL-1β was then analyzed by western blot.

Immunoprecipitation

After cell stimulation, cells lysates were collected to test the interaction using low salt lysis buffer with protease and phosphatase inhibitors. Place the lysates on the shaker in 4 degree for 20 min to make sure the proteins are completely released into the buffer. Spin down the cell debris or other reagents by centrifugation at 12,000 g for 5 minutes. The supernatant were collected and used for protein concentration quantification. These were pre-washed with the beads with secondary antibody of choice. They were tumbled at 4 ℃ for 30 minutes. Then remove the washing supernatant and add primary antibody to the tubes. This was tumbled overnight at 4 ℃. On the second day, the secondary antibody was then added and tumbled for 2 hours at 4 ℃. This was spun down at 8,200 g 5min. Then remove the supernatant, leaving the agarose

beads. The left beads were resuspended in 300µl of low salt lysis buffer to wash and spun down at 8,200 g. Repeat for two times for a total of three washes. Finally the agrose beads were spun down with the supernatant removed. 15 µl of 4xSDS protein loading buffer was added to each sample for SDS-PAGE gel running.

siRNA mediated knockdown by electroporation

Add the entire tube of supplement solution into Nucleofector® Solution. Pre-load 12-well plates with 1 ml of indicated culture media. Pre-warm the medium within the plates in a 37 ℃/5% CO2 incubator. To prepare the cells, quantify a small aliquot of the cells to determine the cell density. Prepare 1 x 10^6 cells for each sample, and centrifuge the cells at 90xg for 10 minutes at room temperature. Remove cell supernatant thoroughly and resuspend the cell pellet with 100 µl of room-temperature Nucleofector® Solution for each sample. Handle the experiment quickly enough to avoid exposing the cells in Nucleofector® Solution for more than twenty minutes so that the cell viability and transfection efficiency are not affected. Add 0.5 µg DNA or 30-300 nM siRNA into the cells suspension with nucleofector solution. Load the mixture into certified cuvette to cover the bottom of the cuvette and make sure the liquid is without air bubbles, and close the lid of the cuvette. Use specifically Nucleofector® Program U-001 for high viability or V-001 for high expression level. Insert the loaded cuvette into the Nucleofector® Cuvette Holder and run the desired program pressing the X-button. After taking out the cuvette, immediately add ~500 µl of the pre-warmed culture medium to the cuvette and gently transfer the cells into the prepared plate using the supplied pipetes. THP-1 cells

can be differentiated in differentiation medium immediately after Nucleofection®. Incubate the cells in 37℃/5% CO2 incubator until analysis. Cells would become adherent one to three days after PMA treatment.

NLRP3 inflammasome activation

In 293-ASC-caspase-1-IL1β cells system, NLRP3 inflammasome was activated by over-expression of adaptor protein NLRP3, which would lead to oligomerization of ASC, activate caspase-1 and cleave pro-IL-1β. Cell lysates and cell supernatants were collected to determine the caspase-1 activity and the IL-1β release level in the supernatant. THP-1 cells are treated with PMA (10 ng/mL) overnight for differentiation in 6 well-plate at 2M cells per well. THP-1 cells must be subculture before the density reaches 1M/ml. The cells are sub cultured to a density between 0.3-0.5 M/ml. The medium need to be changed every 2 to 3 days. On the stimulation day, THP-1 macrophages are first primed with LPS (100 ng/mL) for 3 h. After priming, the cells were washed with PBS for two times to remove the residue of LPS. For stimulation, dilute different ligands using serum free medium (OPTI-MEM) to stimulate the cells for different time points as the experiment designed. Silica (500 ug/ml), MSU (500 ug/mL), CPPD (500 ug/mL) were used to stimulate NLRP3 inflammasome by 6 h stimulation. ATP (5mM), Nigericin (5 uM) were used for NLRP3 inflammasome activation by 1 h treatment.

Results

Screening of DDX family proteins for NLRP3 inflammasome activation

NLRP3 inflammasome can be activated by various categories of inducers. These ligands vary from pathogenic compartments to sterile stimulations. We hypothesized that NLRP3 acts as an adaptor of the signaling by sensing the upstream signals from specific sensor of different ligands, and interacting with ASC to form inflammasome. DDX family proteins contain RNA helicase domain, thus we suspect that DDX family proteins might play a role in NLRP3 inflammasome activation utilizing their DNA/RNA binding domain to sense, for example, DNA or RNA ligands from either dying cells or pathogens. We purchased the cDNA library for gateway cloning system, and constructed cDNA of DDX family genes into tagged pcDNA3.1 vector. After maxi-prep and purification of the plasmids, we transfected the plasmids into 293T cells so that the protein expression and the correct size of the expressed protein were validated. The next step is to test the role of DDX proteins in inflammasome activation. Here, we utilized a 293T engineered cell line which stably over-expresses ASC, pro-caspase-1 and pro-IL-1β reconstituting the inflammasome activation moduole. These genes were transduced into the cells using lenti-virus that packaging the expressing DNA fragment of the indicated genes. For the screening, we co-transfected NLRP3 plasmid with different DDX expressing plasmids into the 293 engineered cells. The medium were changed 24 h after transfection. And the cell supernatants were collected 48 hours after transfection.

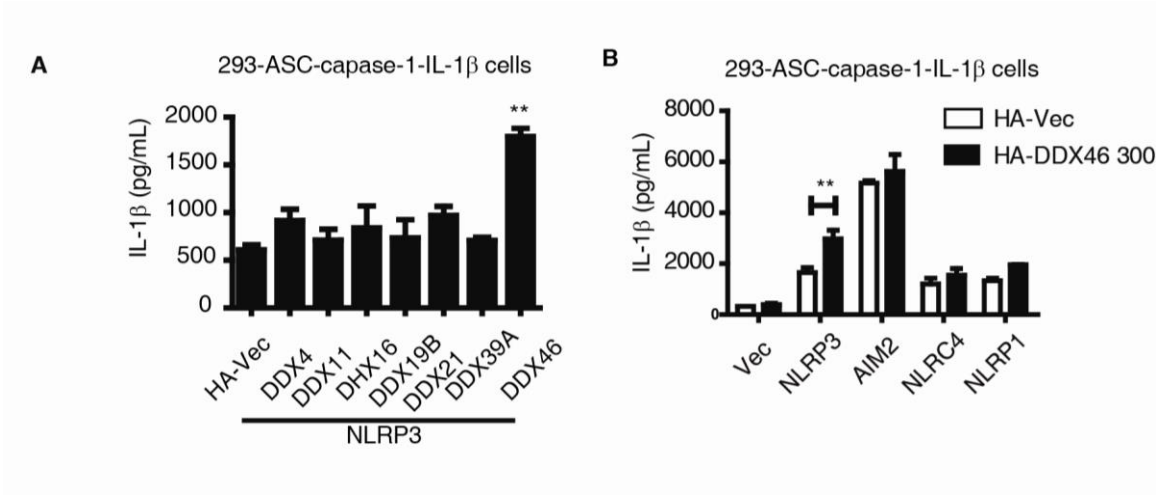


Figure 19. Screening of DDX family in cellular reconstituted inflammasome system. (**A**) 293-ASC-caspse-1-IL-1β cells were transfected with NLRP3 with or without HA empty vector, HA-tagged DDX4, DDX11, DHX16, DDX19B, DDX21, DDX39A and DDX46. Cell supernatants at 48 h after transfection were collected to measure IL-1β release by ELISA. (**B**) 293-ASC-caspase-1-IL-1β cells were transfected with control vector or HA-DDX46, and co-transfected with NLRP3, AIM2, NLRC4 or NLRP1. Cell supernatants at 48 h after transfection were tested for IL-1β release using ELISA.

We found that among these DDX proteins, DDX46 enhanced caspase-1 activation and IL-1β release detected by ELISA (Figure 19).

Over-expression of DDX46 in 293T cells activates NLRP3 inflammasome

NLRP3 inflammasome is known to be activated by multiple sources of ligands, which vary from pathogen derived molecules such as viral RNA, viral DNA and bacterial toxins, to sterile inducers such as ATP, Alum, and silica. We addressed the question that how the innate immune system senses different type of inducers, and transmits these activating signals down to the inflammasome effectors, which activate the caspase-1 and lead to the cleavage of pro-IL-1β and pro-IL-18. Researchers have found several DNA sensors able to detect DNA and activate NF-κB or inflammasome pathway(Brodsky, 2013; Parvatiyar et al., 2012; Zhang et al., 2011). From our preliminary screening, we found that DDX46 may play an important role in promoting NLRP3 inflammasome activity in 293T engineered cell system. To validate the function of DDX46, we first transfected 293-ASC-caspase-1-IL-1β cells with different amount of DDX46 expressing plasmid. We found that DDX46 overexpression alone did not activate pro-caspase-1 cleavage. We co-transfected NLRP3 with empty vector or dose gradient of DDX46, and we found that NLRP3 over-expression enhanced the caspase-1 activation. More importantly, we observed that co-transfection of DDX46 and NLPR3, further enhanced caspase-1 activation to an extent significantly higher than that with NLRP3 alone. And this enhancement is dependent on the dose of DDX46 transfection

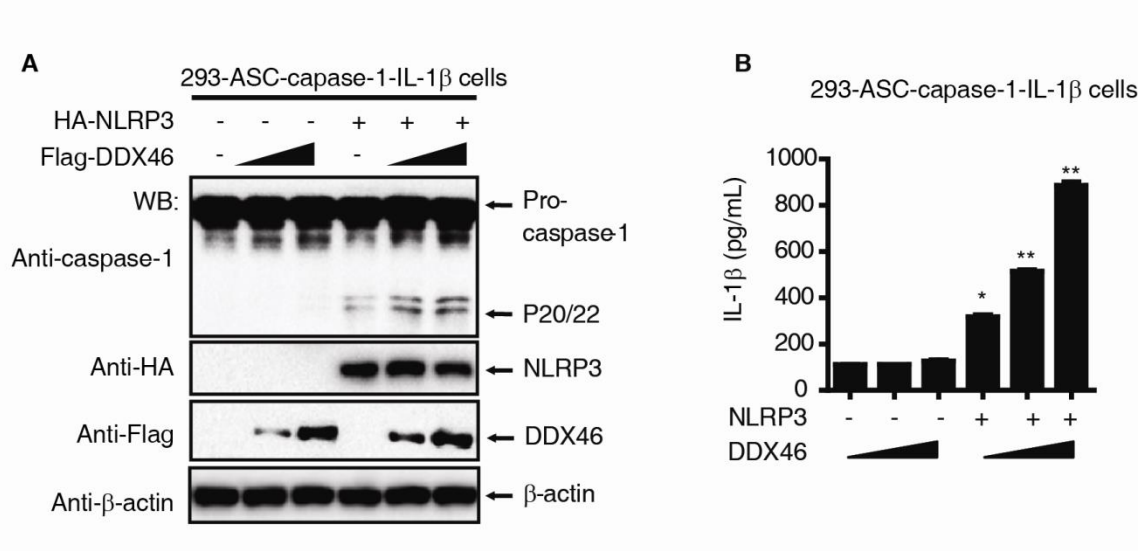


Figure 20. Exogenous expression of DDX46 enhances NLRP3 inflammasome. (**A**) 293-ASC-caspase-1-IL-1β cells were transfected with or without HA-NLRP3 plasmid together with dose gradient of Flag-DDX46 plasmid. Cell lysates were collected using low salt lysis buffer, and the activation of caspase-1 were tested by western blot to check the blot of cleaved caspase-1, P20. (**B**) Cell supernatant from the transfected cells in (**A**) were collected, and the release of human IL-1β were measured by ELISA.

(Figure 20). Using the same system, we measured IL-1β release in the cell supernatants and found that co-transfection of DDX46 and NLRP3 increased the IL-1β release in response of NLRP3 inflammasome activation, further supported the statement that exogenous expression of DDX46 in 293-ASC-capsase-1-IL-1β cells enhances NLRP3 inflammasome.

Knockdown of endogenous DDX46 in 293T-ASC-capase-1-IL-1β cells decreased NLRP3 inflammasome activity

Now that overexpression of DDX46 increased caspase-1 activation in 293T-ASC-caspase-1-IL-1β cells transfected with NLRP3. However, exogenous over-expression of proteins may modify the original function of a protein. Thus, a method to decrease or abort the expression of endogenous expression of the gene is needed to verify the statement. We picked up DDX46 shRNA from the shRNA library purchased from Addgene. shRNA knockdown efficiency was first tested in 293T cells by transfecting the DDX46 expressing plasmid and DDX46 shRNA and detecting DDX46 protein expression by western blot. We picked the shRNA with the highest efficiency for DDX46 knowckdown in 293T cells. Using this shRNA, we knocked down DDX46 in 293T-ASC-caspse-1-IL-1β cells, and transfected the cells with NLRP3, cell lysates 48 hour after transfection were analyzed for caspase-1 activation. When the cells were transfected with DDX46 shRNA, the activated caspase-1 reduced significantly comparing to the cells transfected with control shRNA (Figure 21A). When we looked

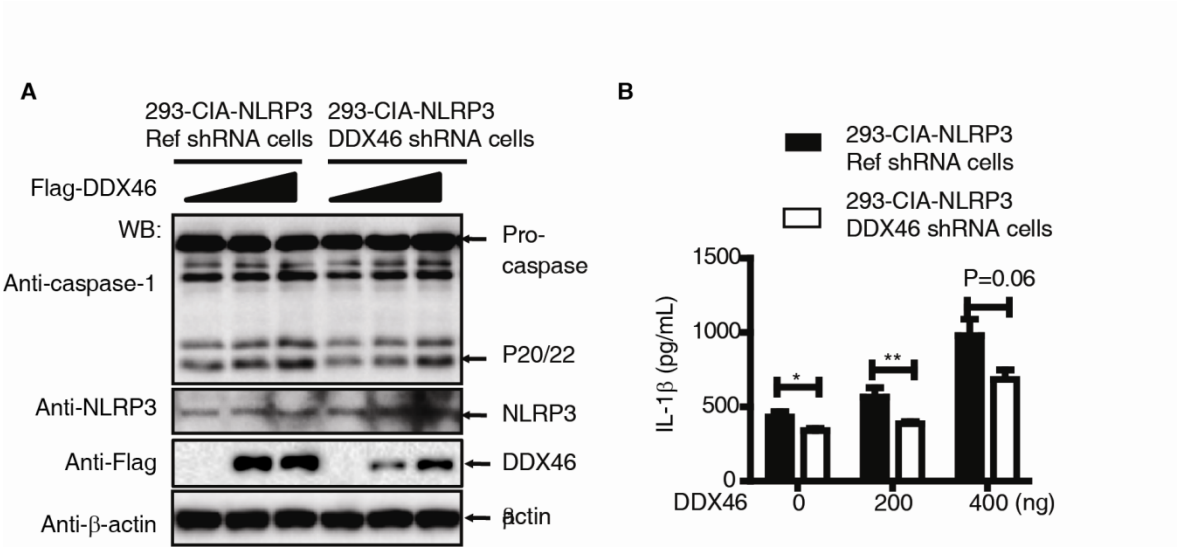


Figure 21. DDX46 knockdown reduced the NLRP3 inflammasome in 293-ASC-caspase-1-IL-1β cells. (**A**) 293-ASC-caspase-1-IL1β cells were transduced with lentivirus to stably express NLRP3. The generated stable cells were co-transfected with dose-gradient Flag-DDX46 and control shRNA or DDX46 shRNA. Cell lysates at 48 h after co-transfection were collected and the activated caspase-1 was tested by western blot. (**B**) Cell supernatants of the transfected cells in (**A**) were collected, and the cytokine level of IL-1β was tested using ELISA.

into the IL-1β release in the supernatant, we also observed a decreased secretion level of mature IL-1β when the DDX46 was knocked down using DDX46 shRNA (Figure 21B).

Knockdown of DDX46 in THP-1 cells reduce inflammasome activity after crystalline stimulations

THP-1, derived from a patient with acute monocytic leukemia, is a human monocytic cell line. It is widely used as a model system for innate immune responses in human immune cells. THP1 cells are usually differentiated into THP-1 macrophages using PMA (50 nM). However, we concerned that high dose of PMA may affect the immune response of THP-1 macrophages or the cells may already be activated when treated with high dose of PMA. We looked up the literature, and found that one group tested the immune response of THP-1 macrophages after differentiation using different dose of PMA, and they found that 10 nM of PMA is effective for differentiation into human macrophages and low enough to avoid impact on immune responses(Park et al., 2007). We also tested the condition, and found that the 5 nM is the good concentration of THP-1 differentiation.

Different dose of DDX46 siRNA was tested to reach the highest knockdown efficiency in THP-1 cells. Aiming to maintain the highest viability of transfected THP-1 cells, I used electroporation protocol with a high survival rate, while use higher dose of siRNA to improve the efficiency. DDX46 knockdown were validated by Real-time PCR using the total mRNA, and the knockdown efficiency was shown to be as high as 80% for DDX46 (Figure 22A). THP-1 cells were first transfected with control or DDX46

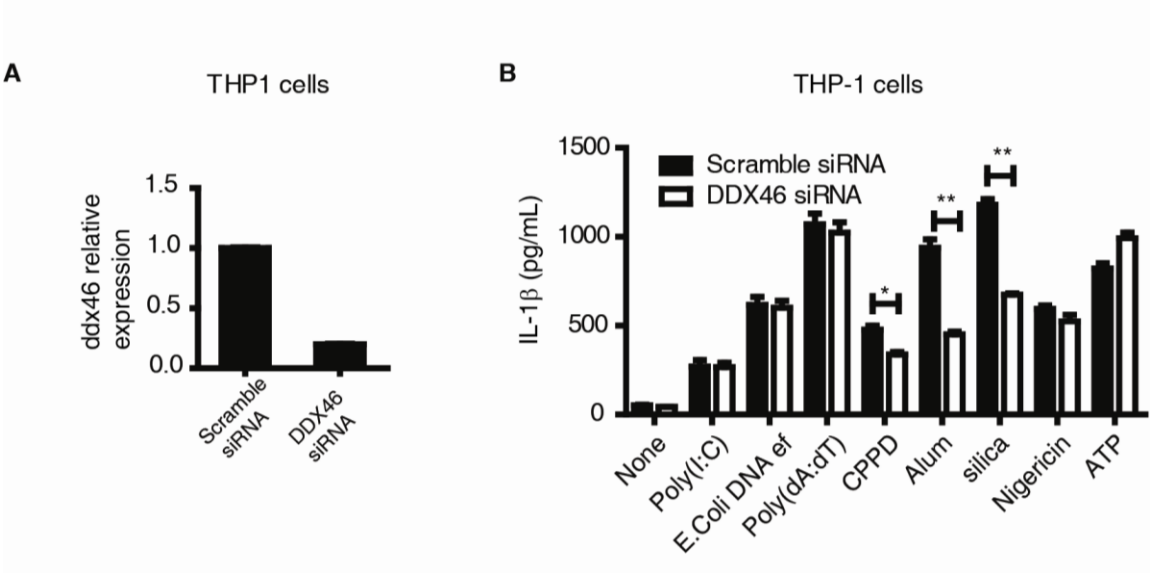


Figure 22. Knockdown of DDX46 reduced crystal induced IL-1β release in THP-1 cells. (**A**) THP-1 cells were transfected with scramble siRNA or DDX46 siRNA using nucleofector kit V by electroporation. Cells were incubated in culture medium with PMA (10ng/ml) for 20 h after electroporation. Total RNA were collected for Real-time PCR. (**B**) THP-1 cells were transfected with scramble siRNA or DDX46 siRNA as mentioned in (**A**), after recovery and differentiation in the culture medium with PMA for 20 h, the cells were first primed with LPS (100 ng/ml), and then after wash with PBS, the cells were treated with multiple inflammasome inducers as indicated for 6 h, or 1h for ATP and nigerine. Cells supernatant were collected to measure the IL-1β release after stimulations.

siRNA by electroporation, and then seeded with PMA (5 nM) overnight for differentiation. On the second day, the differentiate cells were first primed with LPS for 3 hours to initiate the transcription of NLRP3 and pro-IL-1β. Then, I washed the cells for twice with PBS to remove the residue of LPS to avoid the effects of LPS upon inflammasome activation if there would be. Cells were treated with NLRP3 inflammasome inducers for indicated hours. I tested the mature IL-1β release in the cell supernatant, and I found that DDX46 knockdown using DDX46 siRNA significantly reduced the IL-1β release in response of crystalline stimulations, such as alum, silica CPPD (Figure 22B). However, the IL-1β release in response to other categories of stimulations did not vary between knockdown and control groups, indicating that DDX46 promotes NLRP3 inflammasome specifically in response of crystalline stimulations. This result showed a potential importance of the discovery of the function of DDX46 in inflammasome activation, because crystalline stimulation, for example, alum, has been widely used as adjuvant to enhance the adaptive immunity or T cell activation. In other words, the mechanism how DDX46 senses the crystalline ligands and leads the signals to NLRP3 and ASC may explain the mystery of adjuvant immunity.

DDX46 is ubiquitously expressed and is not induced by multiple stimulations

Considering DDX46 overexpression enhanced NLRP3 inflammasome activity, we hypothesized that DDX46 is specifically expressed in immune cells or tissues, and is induced by pathogen-associated molecule patterns, damage associated molecule patterns

or immune responsive cytokines. We purchased the mRNA samples of different mouse tissues from open-biosystem, and generated the complementary cDNA by reverse transcription. DDX46 mRNA expression was tested by quantitive Real-time PCR. DDX46 is actually ubiquitously expressed in different tissue, such as lung, heart, spleen, testis, brain and other tissues (Figure 23A). To test if the gene expression of DDX46 is induced by stimulations, peritoneal macrophages were collected from B6 WT mice. The cells were treated with LPS, IFN-γ and IFN-β. DDX46 was found not to be induced by any of the stimulation added (Figure 23B). These results indicated that the regulation of NLRP3 inflammasome by DDX46 is not dependent on the regulation of DDX46 expression. Other Mechanisms, for instance, post-transcriptional modification of the protein or conformational change of the molecule leading to functional activity change may contribute to the function of DDX46 in inflammasome activation. What we need to do is to examine the protein of DDX46 to check if the protein is modified or processed after translation before or after ligands stimulation in our THP-1 cells system.

DDX46 is cleaved after crystalline stimulation in THP-1 cells

As DDX46 transcription level is not affected by LPS stimulation, we hypothesized that DDX46 is modified after the cells are treated with the crystalline stimulations, changing the activity of DDX46 to activate NLRP3 inflammasome. To test this hypothesis, primed THP-1 cells were treated with multiple NLPR3 inflammasome inducing stimulations, such as poly (I:C), ATP, MSU, CPPD, silica and alum. Cell

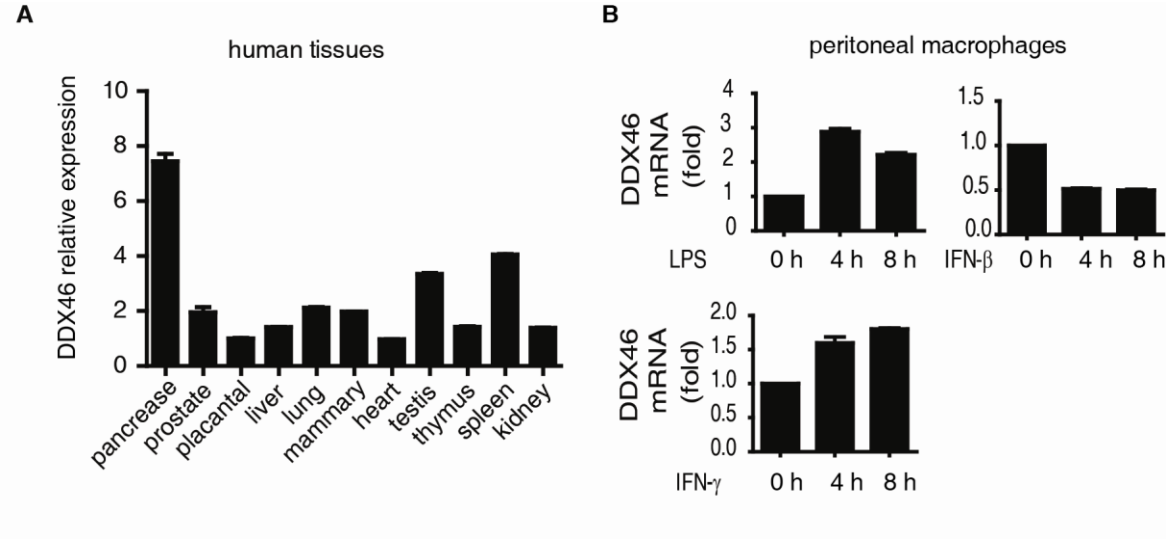


Figure 23. DDX46 is ubiquitously expressed and is not induced by LPS, IFN-γ, or IFN-β. (**A**) Total RNA of different tissues of human being was purchased from open biosystem, and the complementary cDNA were generated using reverse transcriptase. DDX46 expression pattern in different human tissues were determined by Real-time PCR using the cDNA. (**B**) WT peritoneal macrophages were obtained from B6 mice. The cells were treated with LPS (100 ng/ml), IFN-β (10 ng/ml), and IFN-γ (10 ng/ml) for different time points. Total RNA was collected using TRIzol, and the DDX46 mRNA expression were determined by Real-time PCR.

lysates of the cells after stimulation were collected and analyzed for DDX46 protein by western blot. Strikingly, I found that even though DDX46 was ubiquitously expressed after different stimulations, the protein is processed or cleaved into smaller size of protein when the cells were specifically treated with crystalline ligands such as alum and silica (Figure 24). This discovery gave us a light on the mechanism of how DDX46 affect the activation of NLPR3 inflammasome in response of crystalline stimulations. This cleaved form of DDX46 might be important to transduce the danger signal to downstream molecules. There are several possibilities that could explain the function of DDX46. One is that DDX46 can interact with NLRP3 when it is activated. Another possibility is that activated form of DDX46 can bridge the interaction of NLRP3 and ASC to form the platform. Or DDX46 affects the activity of cathepsins which play important role for NLRP3 inflammasome activation after crystalline stimulations. To test these possibilities, we would like to move the system back to 293T cells for molecule mechanisms.

Interaction between NLRP3 and DDX46 in 293T cells

NLRP3 was first found to be important for inflammasome activation by Tschoop's report stating that NLRP3 interacts with ASC and forms a complex or platform which leads to pyroptosis(Agostini et al., 2004). We found that DDX46 affected NLRP3 inflammasome activation in response of silica and alum. Thus, we hypothesized that DDX46 may interact with important components or signaling molecules in inflammasome signaling pathway. Firstly, it would be necessary to check

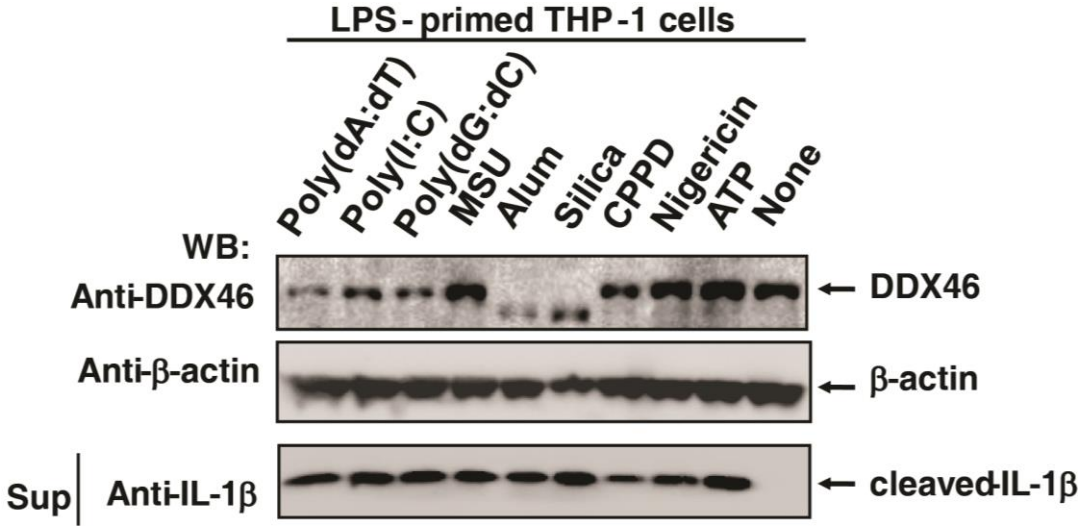


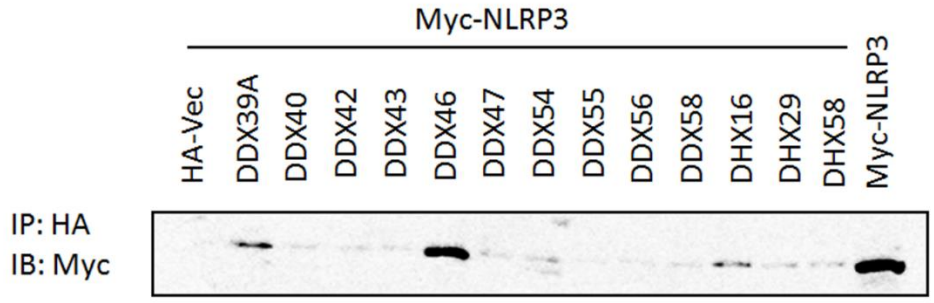
Figure 24. DDX46 is cleaved after silica and alum stimulation. THP-1 cells were primed with LPS for 3 hours, and then washed with PBS for one time. The cells were then transfected with Poly(dA:dT) (1 ug/ml), poly(I:C) (1ug/ml), or poly(dG:dC) (10ug/ml) uing lipofectamin 2000, or treated with MSU (200 ug/ml), alum (500 ug/ml), silica (500 ug/ml), CPPD (200 ug/ml) for 6 h. Other cells were treated with ATP (5 mM) or Nigericin (1 nM) for 1 h. Cell lysates and supernatants were collected for western blot.

if exogenous expression of DDX46 could interact with NLRP3, ASC or other inflammasome compartments. DDX46 and NLRP3 were co-transfected into 293T cells to check the interaction between exogenously expressed DDX46 and NLRP3. Also we checked the interaction between DDX46 and other proteins such as NLRP1 and AIM2 by the same method. HA-tagged DDX46 were co-transfected with Flag-tagged NLRP3, NLRP1, AIM2 or empty-vector in 293T cells. Cell lysates were used for immunoprecipitation using anti-HA beads. Then the pulled down protein lysates were analyzed by western blot and blotted by anti-Flag antibody. We found that DDX46 could interact with NLRP3 but not other proteins such as NLPR1 and AIM2 (Figure 25A). When we over-expressed DDX46 with ASC in 293T cells, we also found interaction between ASC and DDX46 (Figure 25B). Accordingly, we hypothesize that DDX46 may function as an adaptor to bridge the interaction between NLRP3 and ASC.

DDX46 forms a complex with NLRP3 and ASC in THP1 cells after silica stimulation

As in 293T cells, we found that DDX46 over-expression specifically enhanced NLRP3 inflammasome activation, and in THP-1 cells, knockdown of DDX46 leaded to decrease of IL-1β release after NLRP3 inflammasome activation in response of silica stimulation. We already checked that exogenous DDX46 interacts with NLRP3 and ASC in 293T cells, we hypothesized that DDX46 interacts with NLPR3 and ASC in LPS-primed THP1 cells after silica stimulation. Moreover, we were also interested to see when DDX46 starts to interact with NLRP3 or ASC, thus we carried out a time course

A



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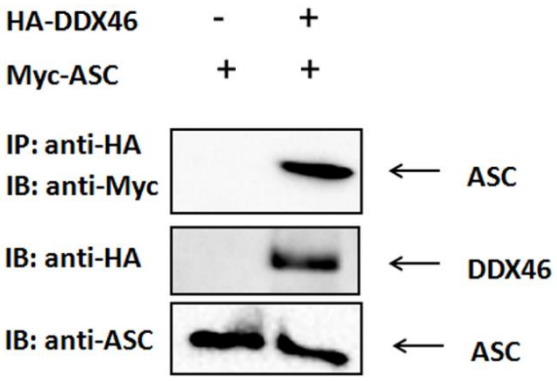

Figure 25. Exogenous DDX46 interacts with NLRP3 and ASC. (**A**) HA-tagged DDX family proteins were co transfected with Myc-NLRP3 in 293T cells. Cell lysates were used for immunoprecipitation to pull down HA-tagged proteins to detect interacted NLRP3 by western blot. (**B**) 293T cells were co-transfected with HA-DDX46 and Myc-ASC. Cell lysates were used for immunoprecipitation to pull down HA-tagged DDX46 and to detect Myc-ASC by western blot.

treatment of silica in LPS-primed THP-1 cells, to detected the interaction by immunoprecipitation. We fist differentiated the THP-1 cells into attaching THP-1 differentiated macrophages with PMA, and primed the cells with LPS for 3 hours on the stimulation day. Cells were then treated with silica for different time points after cell washing with PBS. Cell lysates were collected for immunoprecipitation.

We found that after THP1 cells were primed by LPS, DDX46 started to interact with NLRP3, and this interaction did not significantly increased when the cells were treated with silica for 2 or 3 hours. On the other hand, DDX46 does not interact with ASC in absent of silica stimulation, but DDX46 starts to interact with ASC at 2 h after stimulation and strongly interacts with ASC at 3 h after stimulation, when DDX46 is strongly interacted with NLRP3 (Figure 26). Notably, DDX46 was cleaved at 3 h after silica stimulation, suggesting that cleaved form of DDX46 has stronger ability to interact with ASC, bringing ASC to NLRP3 for inflammasome platform formation. We also checked the interaction between NLRP3 and ASC after silica stimulation. We found that NLRP3 did not interact with ASC at the early time points, but strongly interacted with ASC at 3 hours after stimulation, which supported the hypothesis that DDX46 is cleaved after silica stimulation, and cleaved DDX46 bridges the interaction between NLRP3 and ASC for inflammasome activation. These results provided bases for our working model in which DDX46 bring NLRP3 to ASC for pyroptosis platform formation in response of silica stimulation.

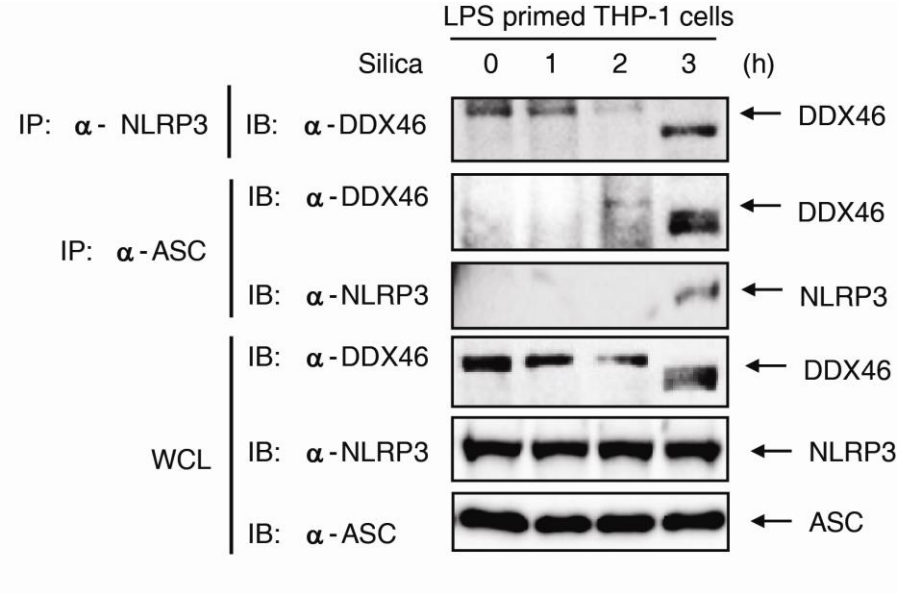

Figure 26. Cleaved DDX46 forms a complex with NLRP3 and ASC. THP-1 cells were primed with LPS for 3 h, and then the cells were treated with silica (500 ug/ml) for indicated time points after wash with PBS. Cell lysates were collected for immunoprecipitation and the interaction between ASC or NLRP3 with DDX46 were detected by western blot.

DDX46 promotes the interaction between NLRP3 and ASC after silica stimulation in THP-1 cells

Regarding the fact that DDX46 interacts with the complex involving NLRP3 and ASC at least 3 hours after stimulation, we hypothesized that DDX46 bridges the interaction between NLRP3 and ASC after silica stimulation. To verify this hypothesis, it is important to remove the function of DDX46 and check the immune-response of the cells without DDX46 or with low level of DDX46 protein. The method we used is to knockdown the DDX46 in THP-1 cells by transfecting the cells with siRNA targeting DDX46 with low off-target. We transfected the THP-1 cells with DDX46 siRNA using electroporation, and checked the expression of inflammasome related protein expression. DDX46 was efficiently reduced by siRNA transfection. And the expression level of pro-caspase-1, ASC and NLRP3 were comparable between the group transfected with DDX46 siRNA and that with control siRNA (Figure 27A). The transfected cells were differentiated into macrophages and primed with LPS to initiate the NLRP3 and pro-IL-1β expression. Then, the cells were treated with silica for 3 hour, a time point we know that NLRP3 strongly interact with ASC. We carried out immunoprecipitation using the cell lysates to check the interaction between ASC and NLRP3 after stimulation in these two groups that were transfected with DDX46 siRNA and control siRNA respectively. The western blot showed that the interaction between NLRP3 and ASC was markedly reduced when DDX46 was knockdown in the THP-1 cells (Figure 27B), while the expression of ASC and NLRP3 are similar between these two groups. In sum, DDX46

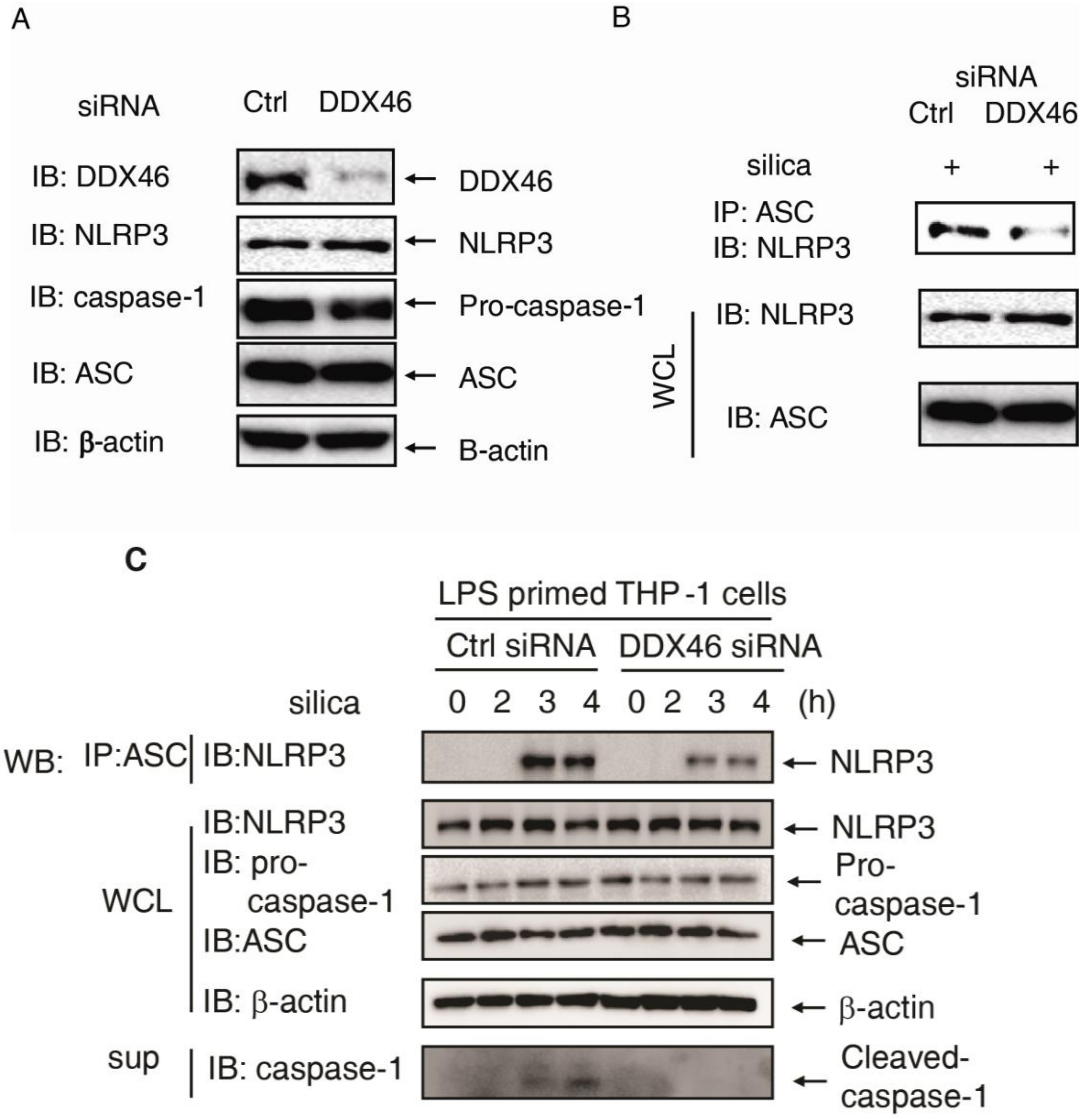

Figure 27. DDX46 knockdown reduced the interaction between NLRP3 and ASC after silica stimulation. (**A**) THP-1 cells were transfected with control siRNA or DDX46 siRNA by electroporation. Cell lysates were collected for western blot. (**B,C**) THP-1 cells were transfected with control siRNA or DDX46 siRNA, and cultured in PMA containing medium for 20 h. Cells were then primed with LPS and treated with silica for 3 h (**B**) or indicated hours (**C**). Cell lysates were collected after stimulation and used for immunoprecipitation.

plays an important role in resulting in the interaction between NLRP3 and ASC in response of silica stimulation. It can explain why knockdown of DDX46 reduced the IL-1β release in response of crystalline stimulations in THP-1 cells. We also did a time course to detect the interaction between NLRP3 and ASC after silica stimulation in cells transfected with DDX46 siRNA. Consistently, we found that knockdown of DDX46 by siRNA reduced the interaction between NLRP3 and ASC at 3 h and 4 h after silica stimulation (Figure 27C). In addition, the active caspase-1 in the supernatant also decreased in the DDX46 knockdown cells.

DDX46 C-terminal deletion has stronger ability to stimulate NLRP3 inflammasome in 293T-ASC-caspase-1-IL-1β cells

As we found that DDX46 was cleaved at 3 hour after silica stimulation in THP-1 cells, co-existing with the occurrence of the interaction between NLRP3 and ASC, and the cleaved DDX46 also interacted with NLRP3 and ASC at 3 hour after silica stimulation. We suspected that cleaved DDX46 has physiological function to activate NLRP3 inflammasome. Here we go back to 293T engineered cell system to work on the molecule mechanism of the function of DDX46. I designed primers and amplified the cDNA that only express amino acids from the first one to the 992 th amino acid, a fragment that only express the truncation of the DDX46 protein lacking the C-terminus, and I referred this protein as DDX46 ΔC. Utilizing the 293T engineered system, I transfected the cells with NLRP3 and then co-transfected with DDX46 FL plasmid or

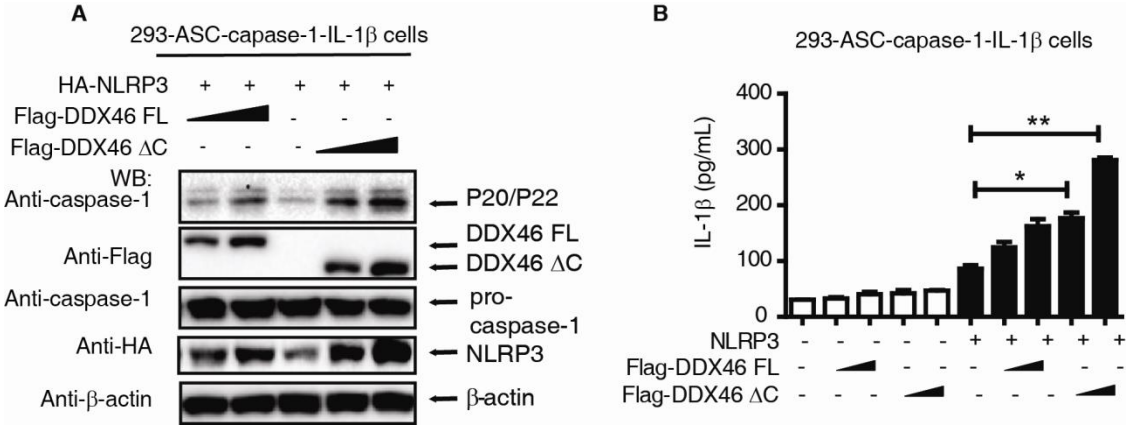

Figure 28. DDX46 ΔC has stronger ability to promote NLRP3 inflammasome. (**A**) 293-ASC-caspase-1-IL-1β cells were co-transfected with NLRP3 and DDX46 or DDX46 DC of different doses. Cells lysates collected 24 h after transfection were analyzed for caspase-1 activation by western blot. (**B**) Cell supernatants of the transfected cells in (**A**) were analyzed for IL-1β release by ELISA.

DDX46 ΔC plasmid. When I examined the caspase-1 cleavage of the cells using the cell lysates, I found that even though DDX46 full length itself enhanced the NLRP3 inflammasome activity by increasing the pro-caspase-1 cleavage, DDX46 ΔC further enhanced the inflammasome activity to higher extent by showing significantly stronger signal of P20 of caspase-1 (Figure 28A). Similarly, we measured the secretion of human IL-1β, and consistently we found that DDX46 ΔC co-transfection led to a significantly higher level of IL-1β release compared to that transfected with DDX46 full length gene (Figure 28B). This result indicated that the cleaved form of DDX46 after silica stimulation has got a physiological function to enhance the NLRP3 inflammasome, but the full length form of DDX46 does not have the activity to stimulate NLRP3 inflammasome.

Exogenous DDX46 ΔC interacts with NLRP3 and strongly with ASC

In the THP-1 cells we found that cleaved DDX46 can interact with the complex formed by NLRP3 and ASC 3 hours after silica stimulation. Thus, it would be interesting to check if exogenous DDX46 ΔC has the activity to interact with NLRP3 or ASC. I transfected 293T cells with Flag-tagged DDX46 or DDX46 ΔC and HA-tagged NLRP3, cell lysates were collected 24 hours after transfection to test the interaction between overexpressed DDX46 and NLRP3. By incubating the lysates with anti-HA beads, the overexpressed NLRP3 and its potential interacting molecules in the cell lysate bound to the beads, and was later precipitated through centrifugation with

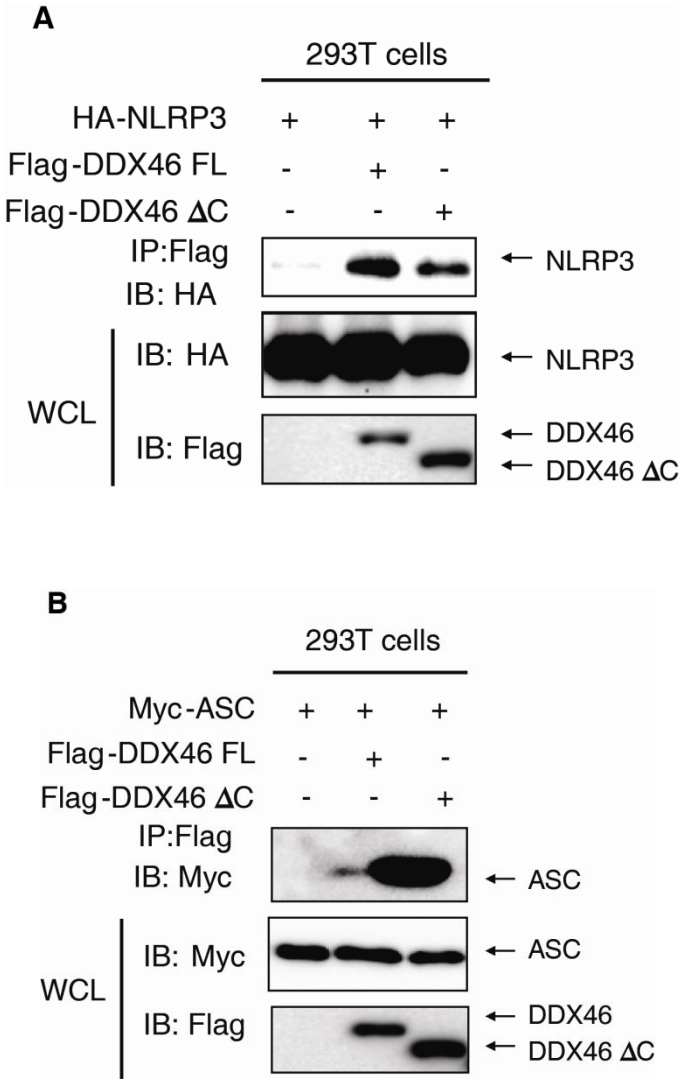

Figure 29. DDX46 ΔC interacts with ASC and NLRP3. (**A**) 293 T cells were transfected with plasmid of DDX46 and DDX46 ΔC along with NLRP3. Cells lysate at 24 h after transfection were analyzed by immunoprecipitation an dwestern blot to detect the interaction between NLRP3 and DDX46 or DDX46 ΔC. (**B**) 293T cells were transfected with DDX46 or DDX46 ΔC with ASC. The interaction between ASC and DDX46 or DDX46 ΔC was determined by immunoprecipitation and western blot.

several wash. Finally the interacted proteins were dissolved into the SDS-loading buffer, which would be ready for western blot after 5 minutes incubation in 100 degree. According to the immunoprecipitation result, the cleaved DDX46 lacking c-terminus and the full length DDX46 can both interact with overexpressed NLRP3 (Figure 29A). Using the same method, I also checked the interaction between DDX46 ΔC with Myc-tagged ASC, and observed that DDX46 ΔC had much stronger ability to interact with exogenous ASC than the full length of DDX46 (Figure 29B). These results were consistent with those I did in THP1 cells, so that we could conclude that DDX46 can constitutively interact with NLRP3 in the cytoplasm, and when itself is cleaved by other proteins as the cells are treated with stimulations such as silica, alum, the activated form of DDX46 could bring ASC to NLRP3 utilizing its strong affinity to interact with ASC, forming a platform formed by oligomerization of NLRP3, ASC and cleaved DDX46. This complex after stimulation finally leads to activation of caspase-1 cleavage.

Caspase-3 cleaves DDX46 after silica stimulation

As the database estimated that DDX46 can be processed by caspases, we are interested to find which caspase is responsible for the cleavage of DDX46 after crystalline stimulation in macrophages. Firstly, we screened several caspases such as caspase3, caspase 4, caspase8 in THP1 cells using siRNAs targeting these caspases to see if the knockdown of specific caspase would have any effect on the cleavage of DDX46 after stimulation. In THP1 cells transfected with the control siRNA, caspase4 siRNA or caspase8 siRNA, DDX46 was cleaved when the cells were treated with silica

for 3 hours, however, in those that transfected with caspase-3 siRNA, DDX46 failed to be cleaved after stimulation. This result indicated that caspase3 may be the effecter to cleave DDX46. For further verification, we move the system to mouse bone marrow derived macrophages. After that, the caspases activity was determined in the BMDMs after silica stimulation. Using the cell lysates of the cells treated with silica for different time points, we found that caspase-3 cleavege was activated 2 hours after silica stimulation, and the activity maintained at 3 and 4 hour after stimulation (Figure 30A). However, caspase-1 activity or cleavage was not detected until the cells were treated for 4 hours. Considering DDX46 is cleaved 3 hour after stimulation, we highly suspected that caspase-3 play the major role to cleave the DDX46. To further test our hypothesis, we ordered B6, caspase-1 knockout, and caspase-3 knockout mice from Jackson' lab. We extracted bone marrow progenitor cells from these mice and have them differentiated into bone marrow derived macrophages. We primed the cells with LPS and treated the cells with silica for different time points. The western blot result showed that DDX46 is cleaved at 4 hour after silica stimulation in both B6 and caspase-1 knockout mice cells, however, caspase-3 knockout cells showed a resistance to DDX46 cleavage. In caspase-3 KO cells the cleavage of DDX46 was abrogated (Figure 30B). It gave strong evidence that DDX46 cleavage relies on the activated caspase-3 after the cells are treated with silica. Besides, caspase-3 deficiency also led to reduce IL-1β secretion at 4 h after silica stimulation (Figure 30C). This finding first links caspase-3 to pyroptosis, a programmed cell death functions through caspase-1 activation, other than

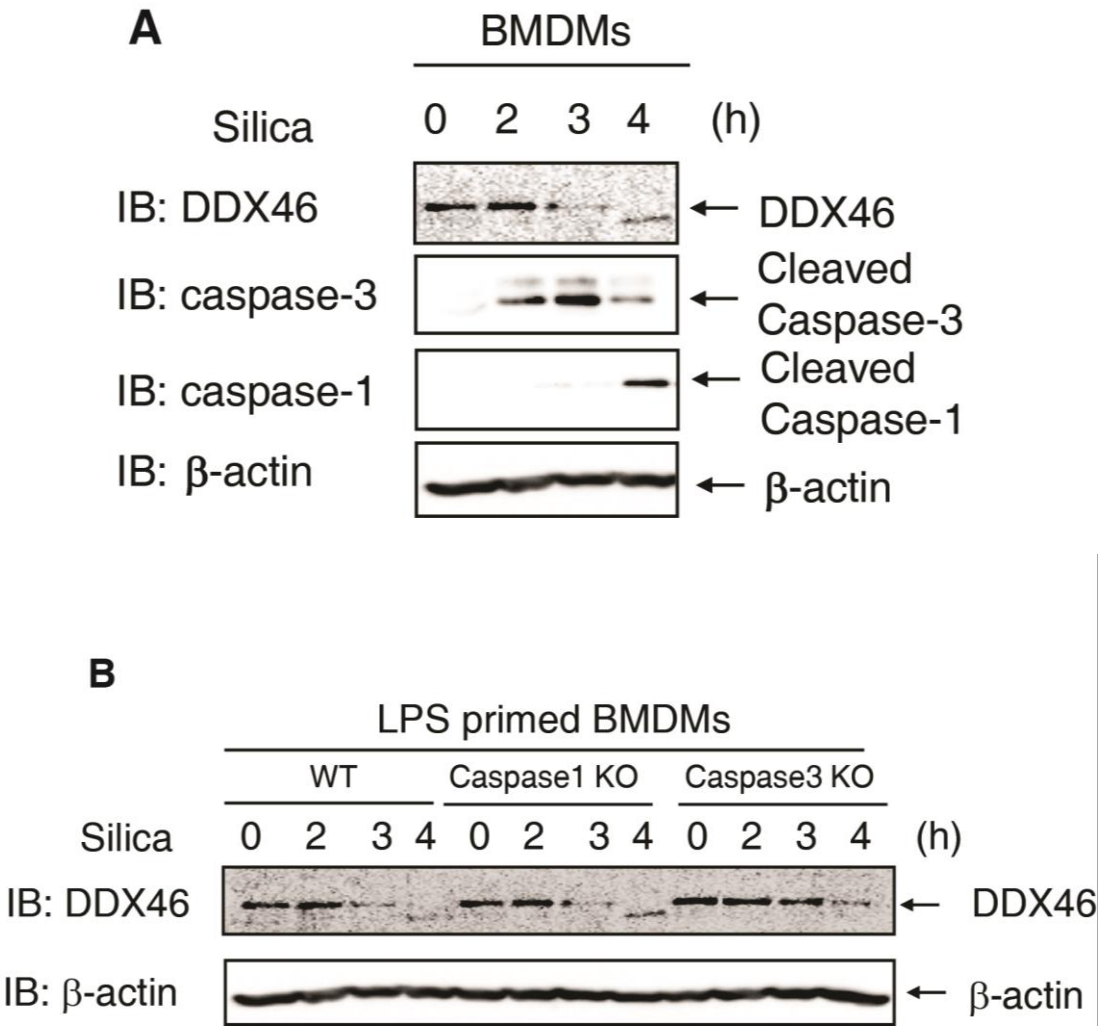

Figure 30. Caspase-3 is essential for DDX46 cleavage after silica stimulation. WT BMDMs were primed with LPS, and then treated with silica for indicated time points. Cell lysates were analyzed by western blot to exam the activation of caspase-1, caspase-3 and DDX46.

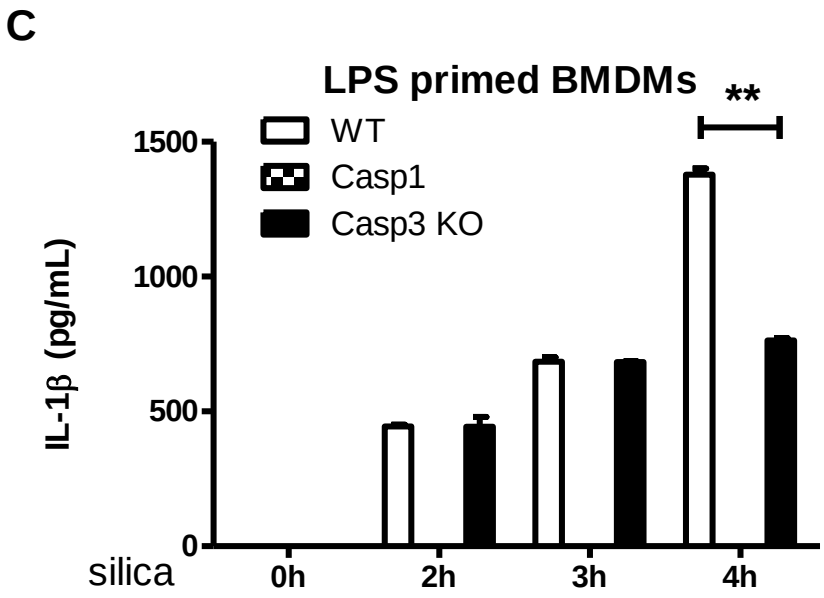

Figure 30. Continued.

apoptosis, providing a new inspect of this protein in the mechanism of programmed cell death, and other potential functions.

Working model of DDX46 leaded NLRP3 inflammasome activation

Based on these results, I generated a cartoon that explains how DDX46 promotes NLRP3 inflammasome under crystalline stimulation (Figure 31). In the NLRP3 expressing cells, DDX46 consistently interacts with NLRP3. When the cells are challenged by crystalline stimulations such as silica, alum, caspase-3 is cleaved and activated, and the activated caspase-3 processes DDX46, leaving the cleaved form of DDX46 interacting with NLRP3. Then, the cleaved DDX46, with its strong ability to interact with ASC, brings the NLRP3 to interact with ASC forming a large complex by oligomerization of these proteins. And the large protein complex, that we called inflammasome, activates caspase-1 cleavage and thus processes pro-IL-1β and pro-IL-18. The cells are finally led to pyroptosis.

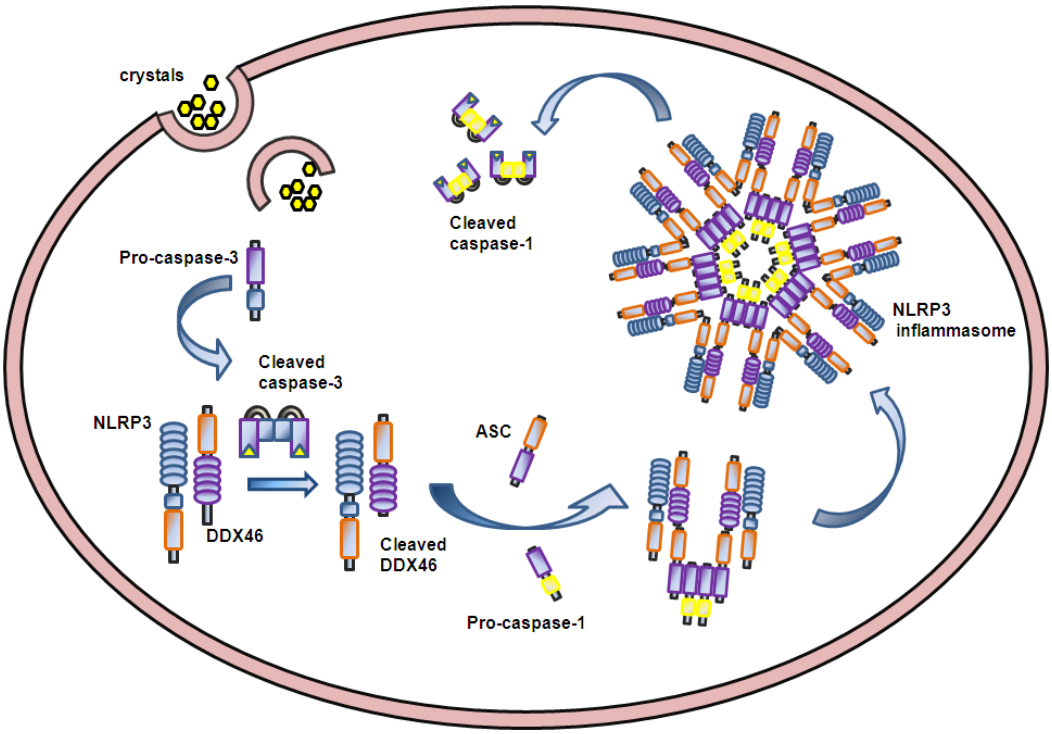

Figure 31. Working model of DDX46 in NLRP3 inflammasome. DDX46 interacts with NLRP3 in the cytoplasm. When the cells are treated with crystals, caspase-3 is activated, and it cleaved DDX46 into a activated form, which could bring ASC to NLRP3 and form a large complex of inflammasome. NLRP3 inflammasome cleaves the pro-caspase-1 and release the activated caspase-1.

Discussion

DDX46 in inflammasome activation

In this study, by screening of DDX and DHX family members, we have identified DDX46 as a critical component of NLRP3 inflammasome activation. This inflammasome is critical for caspase-1. DDX46 binds to both NLRP3 and ASC, and interestingly, DDX46-ASC binding is dramatically enhanced after crystalline ligand stimulation such as silica but not soluble activators such as DNA/RNA ligands or ATP. The molecular mechanism of increased binding may involve a possible caspase-3 dependent cleavage of DDX46 at the C-terminus, eliciting a possible conformation change of the DDX46 molecule and facilitating its binding with ASC. Since DDX46 can bind to NLRP3, the outcome of increased ASC binding upon stimulation resulted in a recruitment of NLRP3 and ASC molecules by DDX46. By bioinformatics prediction, DDX46 has two caspase cleavage sites at the C-terminus, they are 871 site, ESQD-VMQQ and 922 site, ERQD-GGQN. The exact cleavage site needs to be further determined by proteomics analysis. By constructing the ΔC truncation of DDX46, we indeed found that the ability of truncated DDX46 to activate NLRP3 inflammasome is significantly enhanced. The potential molecular mechanism of DDX46 is bridging the interaction between NLRP3 and ASC by the truncated form because the DDX46 ΔC strongly interacts with ASC whereas the full length does not. Finally, by using caspase-3 KO mice, we found that caspase-3 activation is needed for crystalline induced DDX46 processing. It is reported that crystalline stimulation such as silica stimulation will cause

apoptosis and necrosis of cells (Joshi and Knecht, 2013). Such necrosis can serve as a trigger for inflammasome activation (Li et al., 2009). The cleavage of DDX46 in this case acts as a sensor of the necrosis danger signals and activates the NLRP3 inflammasome. Indeed, caspase-3 knockout BMDMs produced much less IL-1β upon silica stimulation *in vitro* suggesting that the danger signals induced DDX46 cleavage is very important in inflammasome activation and may be potentially critical immune responses *in vivo*. The physiological role of DDX46 in inflammasome activation *in vivo* and its sequential importance for adequate immune responses upon crystalline stimulation *in vivo* such as in rheumatoid arthritis remain to be determined by constructing a genetic KO model.

Recently, some of the DDX family members have been identified to play important role in type I interferon pathway by directly sensing the ligands or by recognizing the secondary messengers. For instance, DDX41 is a sensor for intracellular DNA and activates type I IFN signaling through STING in the system built up with dendritic cells (Zhang et al., 2011) . DDX41 was also shown to recognize the bacterial secondary messengers cyclic di-GMP and cyclic di-AMP to activate type I interferon immune response (Parvatiyar et al., 2012). We focused on what DDX or DHX family members may function to promote NLRP3 inflammasome activation by sensing the stimuli or downstream signals (like secondary messengers). Among the members screened *in vitro* by 293-CIA cells, we identified a positive regulator of inflammasome activation, DDX46. It was recently reported that a DExD/H-box helicase superfamily member DHX33, a RNA sensor uses NLRP3 to activate the secretion of

proinflammatory cytokines. These data strongly suggested that the DExD/H-box helicase superfamily is highly utilized by the innate immune system for sensing viral infection, bacterial infection as well as danger signals such as crystalline stimulation to activate the inflammasome pathways.

It has been shown that the NLR family members including NOD1, NOD2, NLRC4, NLRP1 and NLRP3 are playing critical roles in linking signals from PAMPs and DAMPs to the activation of cytosolic inflammatory responses. Our study shows one important molecular mechanism of how the assembly of NLRP3 inflammasome platform with ASC is formed. Recent studies showed that DHX33 or NAIP senses specific ligands and interacts with their adaptors NLRP3 and NLRC4 respectively to trigger inflammasome activation(Brodsky, 2013; Mitoma et al., 2013). It is still not clear what could be the sensors of crystalline stimulators such as silica and alum, which are proved to be very critical causes of lung inflammation and adjuvant respectively. Some crystalline stimulus such as uric acid crystals can cause gout by inflammasome activation at the joints. It is found that these crystallines have common features of causing the release of ‘danger signals’ from dying cells. Cell death is usually mediated by some death associated caspases, caspase 3, 6, 7. We found that the death pathway which activates caspase-3 may be a bridge between the danger signal and the activation of inflammasome. Because the activation of caspase-3 can mediate the cleavage of DDX46, rendering a much potent form of the molecule to bridge the interaction between NLRP3 and ASC, two pivotal components of inflammasome platform. Although not a single sensor of crystalline has been successfully identified to activate NLRP3

inflammasome so far, our data provide a molecular basis of how the cell “senses” danger signals from dying cells caused by engulfing the insoluable crystals. Thus, our study may shed new light on crystalline induced pathology and these findings may provide important clues for development of specific and effective therapies for inflammatory diseases such as gout and pneumonoultramicroscopicsilicovolcanokoniosis.

CHAPTER IV

CONCLUSION

In the first part, NLRC5 deficient mice were characterized to verify the function to regulate NF-κB signaling, type I IFN signaling and MHC I expression. We observed that NLRC5 ablation in MEFs and macrophages resulted in promoted NF-κB signaling and pro-inflammatory cytokine secretion level after the cells are treated with TLR ligands. In addition, NLRC5 deficiency increased type I IFN signaling activity as well as anti-rival responses in mouse-differentiated MEFs and mouse macrophages when the cells were challenged with virus or TLR3 agonists. Also, significantly reduced MHC I expression was observed in $NLRC5^{-/-}$ T cells. In summary, NLRC5 negatively regulates TLR ligand-induced NF-κB signaling and type I IFN signaling pathway under physiological conditions in mouse MEFS and macrophages. NLRC5 also positively regulates the expression and presentation of antigen presenting molecule, MHC class I, in lymphocytes.

In the second part, by knocking down DDX46 in THP-1 cells, we found that reduced expression of DDX46 led to decreased interaction between ASC and NLRP3, as well as reduced secretion of mature IL-1β after treatment of crystals such as silica and alum. This result confirmed our finding in 293-ASC-caspase-1-IL-1β cells that DDX46 overexpression enhanced the NLRP3 inflamamsome. Furthermore, DDX46 is cleaved by caspase-3 in response of silica stimulation, and the cleaved DDX46 strongly interacts

with ASC, and brings ASC to NLRP3, which DDX46 constantly interacts with. Thus, cleaved DDX46 enhanced silica induced NLRP3 inflammasome activation.

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